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233. THE EFFECT OF INCREASED IODINE FEEDING UPON THE IODINE CONTENT OF COWS' MILK[1]

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(With 1 Figure)

ACCUMULATING evidence has repeatedly demonstrated the value of adding iodine to the feed of domestic (1,2,3) or of laboratory animals (4,5). Subsequent beneficial effects of a varied nature have been widely noted (6,7,8), while injurious results, unless too great quantities are added, have seldom been described.

Several investigators have observed that the milk iodine increases subsequent to augmenting the food iodine of goats (9) or of dairy cows (10,11). Hanford *et al.* (12) have recently made a similar observation. The effect upon milk production has been, if anything, beneficial (13,14,15,16). Thus, Stiner's (13) observations, made in areas recognized as iodine deficient, demonstrated an increased milk production after iodine was added to the feed of dairy cows. This was corroborated by Scharrer (14) and others (16), who found that goats yield an increased amount of milk following the addition of supplemental iodine to their diet.

In a previous investigation made within our group, it was shown (17) that increasing the iodine fed to Brown Swiss dairy cows results in a definite increase in the blood iodine together with an increased excretion of iodine in the urine, faeces and milk. We were unable to determine any unfavourable effects after 3 years of this regimen, nor have we recognized any subsequently. Consequently, it appeared possible to increase the iodine content of cows' milk, without injury to the cows, by adding iodine to their usual feed.

The value of adding iodine to the ordinary intake of man (18), particularly in those geographic regions known to be iodine deficient (19), has long been recognized. The finding of an effective means of providing these increased amounts of iodine, however, has been a problem. Various procedures, more recently the use of iodized salt, have been instituted. We have found it possible to supply patients with an increased amount of iodine in food form by giving milk with an increased iodine content (25,26).

It is the purpose of this paper to present the principal results of an extensive

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investigation which we have recently completed concerning the effect of prolonged increased iodine feeding in increasing the iodine content of cows' milk. In a subsequent report we shall present the results of feeding this milk to patients in the University Hospital.

Methods

The dairy herd chosen for this feeding experiment was owned by John Schaaf and Company.[1] It produces grade A raw milk for delivery in Columbus, Ohio.

The cows were milked twice daily; from 3.00 to 5.00 a.m. and from 2.00 to 4.00 p.m. During the winter months they were maintained in a large covered stable, while during the warmer months the herd was turned out to pasture. They were tested for tuberculosis, *Br. abortus* and mastitis, and their general condition was otherwise carefully guarded.

During the winter months the herd was fed ensilage, alfalfa (lucerne) hay and a grain mixture. The latter was fed in two forms, one of which contained 3·2 mg. % of iodine, added as potassium iodide. Both grain mixtures were prepared and furnished by the Ubiko Milling Company of Cincinnati, Ohio.[1] During the summer months the herd consumed varying amounts of pasture in addition to the above feeds.

Blood was obtained by vena-puncture. Samples for analysis of the water, ensilage, hay and grain mixtures were taken from the usual daily supplies on those days on which the other studies were made. Milk specimens were collected both from the individual cows and as composite samples. Urine and faeces were collected by trained attendants with as little contamination as possible.

Balance studies were made as approximations of the intake and excretion of iodine during a 24 hr. period. The daily consumption of ensilage, alfalfa hay and of the grain mixture was estimated. The iodine content of representative samples was then determined. From these data the daily iodine intake was calculated. No attempt was made to determine the variable iodine intake from the pasture. The minute amount of iodine in the water intake was determined, and found to be negligible. Milk, urine and faeces were collected during 24 hr. periods. Aliquot parts of the pooled 24 hr. samples were then analysed and the total iodine excretion calculated. All the micro-iodine analyses were made after the method of Matthews *et al.* (20).

Jugular blood was obtained from the entire herd of thirty Holstein and thirty Guernsey cows, and two balance studies were made on six cows of each breed previous to the commencement of increased iodine feeding. Fifteen cows of each breed were then fed the iodized grain mixture, beginning 16 February 1936; while fifteen of each breed were maintained as isolated controls. During the early spring, blood and 24 hr. milk specimens were obtained from the

[1] We express our thanks to Mr Dan Schaaf and his co-workers for their willing assistance; also to the Ubiko Milling Company for its generous co-operation.

entire herd of sixty cows. During a period of 5 months, June to October, composite samples of milk for analysis were obtained three mornings weekly from the cows receiving the iodized feed. One balance study was made in midsummer.

OBSERVATIONS

A summary of the blood iodine studies is presented in Table I. Determinations of the milk iodine of the individual cows appear in Table II. The iodine content of successive composite samples of milk collected from the iodized cows

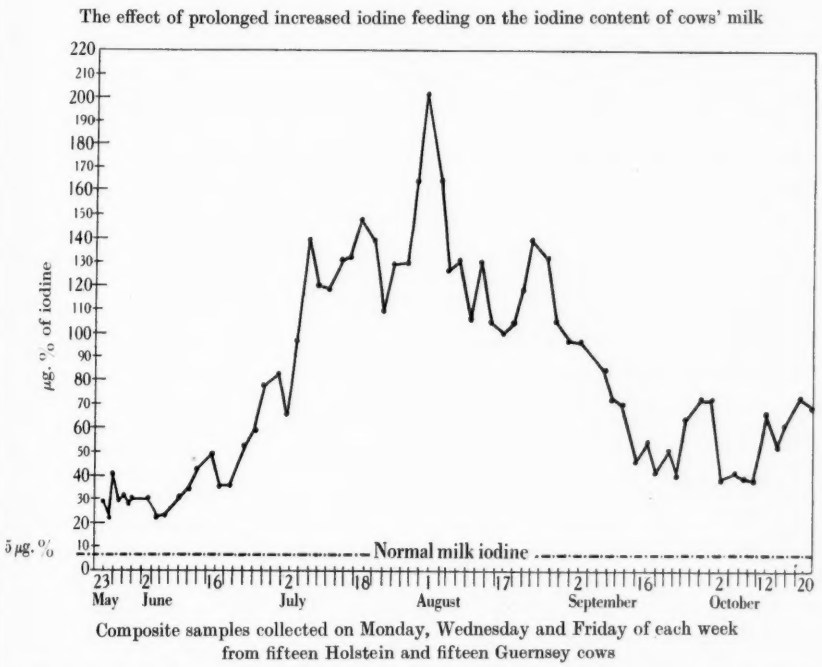

Fig. 1. Note the increasing milk iodine beginning in late June and early July, the summer elevation, and the diminution during the latter part of August and early September.

of the herd during the mid-year is presented in Fig. 1. Results of the balance studies are summarized in Table III, and details of the iodine content of the milk of the animals used in these studies are given in Table IV.

Determinations of the blood iodine of the Holstein cows, previous to the institution of iodine feeding, averaged 4·0 μg. %. Those of the Guernsey cows averaged 3·8 μg. %. Table I shows that in April, after 2 months of increased iodine feeding, the blood iodine of both groups had increased approximately fourteen-fold, to 57·1 and 55·6 respectively. The blood iodine of the uniodized control groups remained normal.

Table II shows that, subsequent to two months of increased iodine feeding,

the increase in the milk iodine was even greater than that in the blood. In April the milk iodine of the control cows was normal, while that of the cows receiving the iodized feed was greatly increased. The Holstein cows showed a twenty-six-fold increase, from 7·6 to 203 μg. %, while the Guernseys revealed a twenty-fold increase, from 7·5 to 149 μg. %. The Holsteins showed the greater

Table I. *Effect of increased iodine feeding on the blood iodine*

Time of test	Breed of cow	Feed fed to group	No. of cows tested	Average blood iodine μg. %	Mean deviation from average μg. %
Nov. 1935	Holstein	Uniodized	26	4·0	0·95
Nov. 1935	Guernsey	Uniodized	27	3·8	0·77
April 1936	Holstein	Uniodized	14	4·9	1·24
April 1936	Holstein	Iodized	13	57·1	12·10
April 1936	Guernsey	Uniodized	8	6·3	2·40
April 1936	Guernsey	Iodized	10	55·6	9·20

Table II. *Effect of increased iodine feeding on the milk iodine*

Time of test	Breed of cow	Feed fed to group	No. of cows tested	Average milk production litres per day	Average concentration of iodine in milk μg. per 100 ml.	Mean deviation from average of milk iodine μg. per 100 ml.	Average daily excretion of iodine in milk mg.
April 1936	Holstein	Uniodized	14	14·0	7·6	2·05	1·04
April 1936	Holstein	Iodized (2 mo.)	11	14·8	203·0	78·00	29·60
April 1936	Guernsey	Uniodized	10	10·6	7·5	1·45	0·69
April 1936	Guernsey	Iodized (2 mo.)	14	8·9	149·0	37·00	12·50

Table III. *Intake and output of iodine of animals used in balance studies*

Date	No. of cows	Breed	mg. of iodine ingested per day (averages)				mg. of iodine excreted per day (averages)			
			Alfalfa hay	Ensilage	Grain mix.	Total	Urine	Milk	Faeces	Total
15. xii. 35	6	Holstein	2·3	8·0	8·4	18·7	2·4	2·7	12·4	17·5
15. xii. 35	6	Guernsey	1·7	6·2	4·7	12·6	5·8	1·1	9·4	16·3
15. ii. 36	6	Holstein	6·0	3·6	0·9	10·5	0·8	0·3	3·4	4·5
15. ii. 36	6	Guernsey	4·5	2·9	0·8	8·2	1·3	0·5	5·2	7·0
17. viii. 36	3	Holstein	6·7	2·2	10·3	19·2	3·1	3·4	21·0	27·7
17. viii. 36	3	Holstein	6·7	2·2	176·6	186·5*	61·0	47·0	113·0	221·0
17. viii. 36	3	Guernsey	5·5	1·6	7·7	14·8	18·3	1·1	10·0	29·4
17. viii. 36	3	Guernsey	5·9	1·6	87·0	94·0*	28·0	5·4	64·0	97·4

* Fed with iodized grain mixture.

Table IV. *Iodine content of milk of animals used in balance studies*

Date	Average iodine intake mg. per day	Average milk values		
		Volume litres	Iodine content μg. %	Total iodine mg.
14. xii. 35	18·7	13·0	24·5	2·7
14. xii. 35	12·6	4·8	20·6	1·1
15. ii. 36	11·0	9·5	3·1	0·3
15. ii. 36	8·2	8·0	8·3	0·5
17. viii. 36	19·1	12·3	29·3	3·6
17. viii. 36	186·0*	21·0	204·0	47·0
17. viii. 36	14·7	7·6	13·1	1·1
17. viii. 36	94·0*	5·0	104·0	5·4

* Fed with iodized grain mixture.

iodine concentration as well as the greater iodine excretion in the milk in this as well as in the August balance study (see Table IV).

Variations in the iodine content of the successive composite milk specimens collected during the mid-year occurred simultaneously with the normal changes in feeding (Fig. 1). During late spring and early summer the pasture was abundant, and the iodized cows were therefore fed less amounts of the grain mixture containing added potassium iodide. This was accompanied by a lowered milk iodine. In midsummer, the grass in the pastures became scarce because of extensive drought. Beginning late in June, the cows were given increased amounts of the grain mixture. The milk iodine again rose to approximately the level attained in April (Table II). During the latter part of August the pasture again improved. However, the feeding of the grain mixture was maintained at approximately the same level. A diminution of the milk iodine was nevertheless evident.

The milk iodine throughout the 5 months averaged 80μg. %. Five determinations made during a corresponding period of 8 months, on cows not receiving the iodized grain, averaged 14·6. Three determinations made 25, 26 and 28 May, on composite samples from cows not receiving the iodized grain were 3·9, 2·4, and 3·6μg. %, respectively.

The balance studies also show that the milk iodine varies with the different levels of iodine intake (Table IV). The total milk iodine output was definitely less than that in the urine and faeces, and similarly depended upon the intake. Table III shows that by far the larger excretion of iodine was in the faeces.

The August balance study shows that the Guernsey and Holstein cows which were given the iodized grain mixture were receiving averages of 94 and 186 mg. per day respectively. The control Guernsey and Holstein cows were receiving averages of 14·7 and 19·1 mg. per day respectively. The pasture and water iodine are not included in these calculations. The water iodine was found to be 0·5μg. %. Calculation of the amount of feed consumed per cow during a year, and of the average iodine content of the feeds, showed that the Guernsey and Holstein cows which were fed with the grain containing added potassium iodide received averages of 106 and 144 mg. of iodine per day respectively. Corresponding values for the control Guernsey and Holstein cows were 15 and 21 mg. per day.

The official dairy tester measured the amounts of feed that the cows were receiving on 16 April, 22 May, 12 June, 17 July, 26 August, 18 September and 15 October. From these observations the average daily iodine received by both Guernsey and Holstein cows in the grain mixture was calculated to be 128, 67, 82, 113, 125, 120 and 118 mg. per day for the given dates respectively.

An attempt was made to determine whether the iodine in the iodized milk was in fat or protein combination. The fat was removed by the Röse-Gottlieb (21) method and the protein by the method of Osborne & Mendel (22). From 80 to 90% of the iodine remained after removal of the fat and protein. In this

respect our observations confirm those of Scharrer & Schwaibold(10), who likewise studied milk from cows fed with relatively large amounts of potassium iodide. They, too, found little iodine in protein or fat combination.

Discussion

The two series of blood iodine determinations made upon the uniodized cows present normal values as obtained by analytical methods employing the most recent improvements(20). They are similar to those obtained in analysing human blood by the same methods. The increased blood iodine of the iodized cows proves the effectiveness of the ingested iodine in maintaining a higher level of iodine available to the individual tissues.

The average iodine content of cows' milk in several goitrous regions has been reported as 2·8μg. %, while that in goitre-free regions is greater, say 6·0μg. %(11). Hanford *et al.*(12) obtained average values of 2·7μg. % in New York, 3·2μg. % in Wisconsin, and 6·9μg. % in South Carolina. In a controlled experiment they found an average throughout the year of 2·5μg. % when feeding an average of 3 mg. of iodine per day. The higher average milk iodine of the uniodized cows in our experiment is in accord with the greater iodine content of the feeds which these cows consumed.

Hanford *et al.*(12) observed a seasonal variation, independent of the diet, in the milk iodine. During periods of indefinite duration in the summer months they obtained milk from control cows maintained on a constant regimen throughout the year. In our three balance studies—December, February, and August—the concentration of milk iodine varied directly with the amount of iodine in the diet.

It would appear that some other factor than the iodine intake must be considered in order to explain the mid-year variation. During the first month of our mid-year study (Fig. 1) the iodized cows produced milk of a lower iodine content than in April. Although part of this decrease was doubtless due to the fact that the cows were fed smaller quantities of the iodized grain during this period, the iodine content of this milk was but little greater than that of cows fed considerably smaller amounts of iodine at other times. The milk iodine of the control cows was likewise low in late May. Furthermore, in late summer and early autumn, when the cows were maintained on approximately the same amount of the iodized grain mixture as in midsummer and in April, the milk nevertheless showed a definite decrease in iodine content. This marked decrease in the milk iodine in our study did not continue throughout the summer, as observed in New York by Hanford *et al.*(12), but became evident during the changes of season; from spring to summer and from summer to autumn. In our study the decrease was found to occur simultaneously with the feeding of abundant pasture. The true nature of this seasonal variation requires further investigation.

The increase in the milk iodine observed in this experiment is greater than that found by other investigators feeding equivalent quantities. Hanford

et al.(12) found milk iodine contents of from 40 to 181 μg. % when feeding 46 and 198 mg. of iodine per day respectively. Orr & Leitch(11) obtained only 33 μg. % by feeding 180 mg. of iodine per day. Scharrer & Schwaibold(10) obtained 29 and 37 μg. % when feeding 100 and 200 mg. per day respectively.

The effectiveness of the use of milk containing increased iodine as a goitre prophylactic has been demonstrated both for man(23) and for experimental animals(24). That a milk of high iodine content can be consistently produced by feeding increased amounts of iodine as potassium iodide has been clearly demonstrated in the present experiment.

SUMMARY

1. The milk and blood iodine of a herd of thirty Guernsey and thirty Holstein cows has been determined, previous to and during prolonged increased iodine feeding to one-half of the herd. The increased amount of iodine was fed as potassium iodide which was mixed uniformly with a grain feed.

2. The blood iodine of the iodized cows was greatly and uniformly increased.

3. The milk iodine was increased. This increase was principally dependent on the amount of iodine which was fed. During late spring, however, the milk iodine from both the iodized and control cows was unusually low. During early autumn the milk iodine from the iodized cows was low.

4. The milk from the iodized cows contained 7–26 times as much iodine as that from the control cows. An average milk iodine of 80 μg. % was obtained from the iodized cows throughout a mid-year period of 5 months.

5. This experiment reveals that a milk of increased iodine content can be readily produced by increased iodine feeding.

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234. BACTERIOPHAGE-ORGANISM RELATIONSHIPS IN THE GROUP OF LACTIC STREPTOCOCCI

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In the course of investigations at this Institute into the part played by lactic streptococci in Cheddar cheese manufacture, it has become very evident that streptococcal bacteriophages are of common occurrence under dairy manufacturing conditions. The main purpose of this paper is to give an account of methods for the isolation of the phages and to describe nine apparently distinct races of phage which have been carefully purified. We do not propose to deal here with the problem of the origin of bacteriophage.

Isolation of phages for lactic streptococci

The cheesemaker uses a culture of lactic streptococci for producing the necessary lactic acid in the milk and subsequently in the curd in the cheese vat. The culture is propagated in sterilized milk from day to day in an amount sufficient to provide 1–2% of clotted culture for use in the cheese milk. Formerly the methods of propagation were very rough and far from aseptic. In recent years, however, more careful methods have been gradually introduced until at the present time a normal bacteriological technique is used in many factories in New Zealand. The system in use has been described in detail previously (1). This improvement in handling has not resulted in immunity from starter trouble. Failures occurred occasionally in the Institute experimental factory until about two years ago, when it was discovered (1) that the use of an increased inoculum eliminated the tendency of cultures to suffer spontaneous failure. This change in cultural conditions has not, however, proved effective universally; in some commercial factories cultures under careful treatment still "die out" or lose their "vitality" (i.e. as acid producers) for no apparent reason within one propagation. In all instances where such failures have been investigated the immediate cause for failure has been found to be the development of a strong concentration of bacteriophage and a coincident lysis of the lactic streptococci. Phage failures may occur in mother culture, bulk culture, or during the development of the starter in the cheese milk and curd. There is even quite commonly an appearance of phage in the whey in a cheese vat where acid is developing normally within the cheese curd. The phenomena are clear cut where a culture of a single strain of streptococci is in use as a starter; they are rather confused where the starter consists of a mixture of several strains because of the partial specificity of the strepto-

coccal phages. In the latter case only one of the strains in the culture may be lysed. Depending on the degree of activity of the lysed strain compared with the activity of the remaining strains the affected culture may or may not show a serious falling off in acid-producing power. It rarely, if ever, fails as completely as does the single-strain culture.

With a lysed or partially lysed single-strain culture it is a simple matter to demonstrate and isolate the phage. A portion of milk culture or of whey taken wherever the failure in acid production has been evident is treated in such a manner as to yield a clear whey (milk can be acidified or treated with rennet and calcium chloride). The whey so obtained is filtered through a sterilized Seitz filter. Drops of a series of dilutions of the fluid in sterile water or saline are then placed on the surface of an agar plate which just previously has been thickly and evenly spread with a clotted milk culture of the *Streptococcus*. Any agar medium which readily supports the growth of lactic streptococci may be used; we use a medium containing peptone, meat extract, sweet whey and yeast extract. The surface of the agar must be thoroughly dried before the culture is spread upon it so that subsequently the drops of diluted whey do not tend to run but are rapidly absorbed. Incubation of the plate at 30° C. overnight usually gives clear-cut results. If phage is present in the whey in a reasonably strong concentration the first few dilutions give clear areas which show up readily against the background of semi-confluent streptococcal growth; the higher dilutions (commonly up to 10^{-7} or 10^{-8}) give progressively decreasing numbers of isolated plaques. Most of the phages encountered have been present in strong concentrations in the original material.

Purification of a phage is carried out by picking an isolated plaque into a tube of sterilized milk, inoculating the tube with a few drops of a culture of the susceptible *Streptococcus* and incubating at 30 or 37° C. until lysis takes place. The process is followed by frequent examination of stained films under the microscope. A trace of the milk from the tube in which lysis has taken place (say one drop of a 10^{-3} dilution) is then added to a flask containing about 500 ml. of sterilized milk; the flask is inoculated with about fifteen drops of a milk culture of the susceptible organism and lysis is again allowed to take place. By this means a large volume of milk containing a strong concentration of the purified phage is obtained. The milk is treated with sterile acid or with sterile calcium chloride solution and heated rennet powder (123° C. for 1 hr.). The clot so formed is broken up and the clear whey which exudes is filtered first through paper and then through a Seitz apparatus. Throughout the final purification process the apparatus used (funnels, filter paper, etc.) must be completely sterile, since the Seitz filter cannot remove contaminant phage. The phage preparation (really the whey containing the phage) can be stored for many months in the refrigerator without much loss in power.

Phages for the lactic streptococci can be propagated on the susceptible organisms growing in broth. The broth first becomes turbid as the organisms start to grow; then the turbidity disappears as lysis takes place. It is much

easier, however, to get good growth of streptococci followed by complete lysis and the development of a strong concentration of phage in milk than in any broth we have so far used.

Where a culture containing a mixture of streptococci is concerned the same principles apply but the procedure is more involved. The phage which is responsible for the failure or sudden decrease in acid-producing activity is likely to be active against only one of the strains present in the mixed flora. It is necessary, therefore, to isolate representative types of streptococci from the culture and to test the original Seitz-filtered whey on the individual cultures. The particular strain on which the phage (if present) acts having been found, the purification procedure is carried out as described above.

General characteristics of the organisms and phages

Eleven strains of lactic streptococci were used as substrate organisms. Nine strains are types of *Str. cremoris* (Orla-Jensen). They form chains of varying length in milk culture, and some of them can be distinguished from one another by the shape and arrangement assumed by the cocci after growth at 37° C. for 6 hr. in sterilized milk. These morphological differences and some more or less constant differences in the rate of acid formation in cheese curd are the only characteristics by which the strains can be distinguished from one another apart from phage action. The sugar reactions of all the strains are practically identical. The total of eleven strains is made up by a stock strain of *Str. lactis* (obtained from Kiel in 1928) and a strain of an "inhibitory" or "non-acid" *Streptococcus*, the characteristics of which have been described by Whitehead (2).

The nine phage races are all very similar in their general properties. They give plaques with a diameter of the order of $\frac{1}{4}$–$\frac{1}{2}$ mm. Their thermal death-points are in the region of 70–75° C. (half-hour exposure at *p*H 6). The different races range in their optimum temperature for action between 30 and 37° C. The only outstanding differences between them lie in their specific action on the various streptococci.

It will be evident at the outset, therefore, that apart from a few points of difference noted above we are dealing with organisms which are distinguishable as separate entities only by the reactions of phages upon them, and with phages which are distinguishable from one another only by their effects upon the various organisms. As will appear later, this leads to difficulties which cannot be resolved until some other basis for differentiation of organisms or phages is found.

Phage-organism relationships

Nine distinct phage races have been carefully purified by repeated picking of plaques into cultures purified several times by the picking of single colonies. The range of activity of the phages against the streptococci is indicated in Table I. A plus sign indicates that the phage lyses the organism on solid and in liquid media and that a titre of at least 10^{-6} can be attained.

Table I. *Phage-organism relationships*

Organisms	Phage races								
	r	*rw*	*k*	*b*	ud_1	ud_5	*ver*	*m*	*lac*
R	+	−	−	+	−	−	−	−	−
RW	−	+	+	+	−	−	−	−	−
K	−	−	+	+	−	−	−	−	−
B	−	−	−	+	−	−	−	−	−
UD_1	−	−	−	−	+	−	−	−	−
UD_5	−	−	−	−	−	+	−	−	−
Ver	−	−	−	−	−	−	+	−	−
M	−	−	−	−	−	−	+	+	−
Cr	−	−	−	−	−	−	+	+	−
Lac	−	−	−	−	−	−	−	−	+
U_9	−	−	−	−	−	−	−	−	+

The plus signs in Table I do not necessarily represent the limits of activity of the phages on the organisms. In several instances a phage has given what appeared to be plaques on a supposedly non-susceptible culture on the surface of agar. No corresponding activity in liquid media could be induced even by addition to the culture of several drops of undiluted phage of high power (as judged by activity against its homologous organism). Burnet(3) mentions similar anomalies between the results on solid and liquid media. Phage *k* at one time reacted doubtfully in this way with culture RW. A year later the action became more frank and it is now possible to propagate phage *k* on RW in milk. In spite of the fact that neither the morphology of RW (which happens to be quite characteristic) nor its acid-producing properties have altered in the meantime, it appears that the organism rather than the phage must have changed since preparations of phage *k* made more than a year ago now act just as strongly on RW as do fresh phage preparations. Owing to this apparent gradual alteration in the organisms the relations between phages and streptococci are not completely static; but the changes are not rapid enough to make the position chaotic.

It will be observed from Table I that there is a marked tendency towards specificity in the range of action of the phage races. As has already been mentioned, there is a fundamental difficulty with the lactic streptococci in making any attempt to classify the phages by their action on the organisms or the organisms by their susceptibility to the phages. The first nine organisms in Table I are all strains of *Str. cremoris*. Where a phage race (e.g. *m*) acts on two strains (M and Cr) it may be merely an indication that the two strains are identical unless another phage is found to act on one of the strains or unless there is an obvious morphological difference between the two strains. Similarly, where two phages act on the same strain or strains of streptococci and thus appear to be identical, it is always possible that another strain susceptible to only one of the phages may subsequently be isolated. These inherent difficulties will most probably be overcome in the future by serological investigations which will, it is hoped, provide independent means for the classification of both organisms and phage races on the basis of antigenic constitution.

With the few organisms and phages given in Table I there appears to be sufficient cross-checking to remove all reasonable doubt of the individuality of both strains of organisms and races of phages except in the case of organisms Cr and M, both of which are attacked by phages *ver* and *m*. The two strains, however, do show a distinct morphological difference.

Our attention has been concentrated almost entirely on *Str. cremoris* because most cheese starter cultures consist mainly of strains of this species. Phage race *lac* active against a stock strain of *Str. lactis* was encountered by chance. In all probability there are just as many phage races for *lactis* strains as for *cremoris* strains. Preliminary trials have shown that phage *lac* does not act upon all strains of *Str. lactis* nor upon other strains of inhibitory streptococci like U_9.

There is ample evidence from the data in Table I, therefore, that susceptibility to phage action is not correlated with some of the other properties of lactic streptococci. Work to be reported later by one of us (G. J. E. H.) has indicated that the sugar reactions of an organism may change while its susceptibility to phage remains constant. The indications are that phage susceptibility depends on some property of the organism entirely distinct from the sugar-fermenting mechanism.

PROPAGATION OF A PHAGE ON DIFFERENT SUBSTRATE ORGANISMS

Since phage *b* readily attacks four organisms (R, RW, K and B) it can be propagated on each one of them as a substrate. The four preparations of phage so obtained have been found to show no constant differences in their action on the four organisms, although in some experiments a phage preparation tends to have a higher titre against the organism on which it has been prepared than against the other three organisms. The most striking feature of the four preparations is that they all act more rapidly and completely at 37 than at 30° C. on all four organisms. This differentiates phage *b* sharply from phages *r*, *rw* and *k*, which each act on one or more of the four strains. Phage *r* on R acts equally well at 30 and 37° C.; phage *rw* on RW acts more rapidly at 30 than at 37° C.; phage *k* on K or on RW acts more rapidly at 30 than at 37° C. There is thus an essential constancy in the character of phage *b*, no matter on what substrate organism it has been prepared. Burnet(4) has emphasized this constancy. He considers that, taken in conjunction with the lack of any simple relationship between a series of organisms and phages, it is strong evidence in favour of a genetic relationship between successive batches of phage.

RESISTANT FORMS

The development of resistant forms in cultures in which lysis has previously occurred follows the normal course described for other groups of organisms. The ease of development of a resistant form and the type of resistance developed is characteristic for each phage race. For instance, phage *rw* acting on strain

RW, on incubation for a further 24–48 hr. at 20° C. after lysis has occurred, readily gives a culture which is completely and permanently resistant to the phage. Phage *r* acting on strain R gives variable results. Sometimes the secondary growth which appears on continued incubation after lysis has occurred is non-resistant; frequently it is resistant but becomes sensitive again after a few subcultures. In some instances further phages, active against resistant strains, have been encountered when attempts have been made to use resistant forms as "starters" in cheese factories (Whitehead & Hunter(5)).

The acid-producing power of resistant forms is very variable. Sometimes the whole resistant culture prepared from an active strain is very low in acid-producing power and is quite useless so far as cheese manufacture is concerned. In other cases the resistant forms are just as active as the original strain. Most commonly there is a mixture of types in the resistant culture. Plating and the picking of individual colonies yields a selection of resistant cultures, some active and some feeble acid producers.

Cross-resistance tests

The reactions between phages, susceptible strains and resistant forms in those cases where the phages act on more than one strain are summarized in Table II. Resistant forms are indicated as follows: R/*b* is the resistant strain developed by the action of phage *b* on strain R. A plus sign indicates that the phage acts readily on the organism growing in milk. A bracketed minus sign indicates that resistance is not permanent. Most probably more of the minus signs in Table II will need to be bracketed as further observations are made.

Table II. *Cross-resistance tests*

	Phage races					
Organisms	*r*	*rw*	*k*	*b*	*ver*	*m*
R	+	.	.	+	.	.
R/*r*	(−)	.	.	+	.	.
R/*b*	+	.	.	−	.	.
RW	.	+	+	+	.	.
RW/*rw*	.	−	−	+	.	.
RW/*k*	.	−	−	+	.	.
RW/*b*	.	+	+	−	.	.
K	.	.	+	+	.	.
K/*k*	.	.	−	−	.	.
K/*b*	.	.	+	−	.	.
M	.	.	.	.	+	+
M/*m*	.	.	.	.	−	−
M/*ver*	.	.	.	.	−	−

It will be observed that cross-resistance tests do not serve to prove or disprove with certainty the identity of two phages. Burnet(6) found this to be so with the Gram-negative bacilli. Thus phages *ver* and *m* both act on strain M; strains M/*m* and M/*ver* are resistant to both phages. If strain Ver were not available (see Table I) it would appear that phages *ver* and *m* were identical. The fact that *m* does not act on strain Ver proves that the two phages are distinct. The same argument applies with phages *rw* and *k*. It follows, therefore,

that a freshly isolated phage which appears identical with (for instance) ud_1 may actually be a different race, the difference becoming demonstrable only if another strain of *Streptococcus* sensitive to one phage but not to the other happens to be found. A polyvalent phage *b* acting on four organisms known to be distinct by reason of their reactions to other phages is less likely to be confused with a freshly isolated phage. A phage *x* which is found to act on exactly the same range of strains attacked by phage *b* is very probably identical with it, although one can never entirely exclude the possibility that some strain of *Streptococcus* sensitive to *x* only or to *b* only will be isolated in the future.

Other types of relationship are apparent in Table II. Organism R is acted upon by *r* and *b*. Strain R/*r* is not resistant to *b*, nor is strain R/*b* resistant to *r*. This, of course, serves to differentiate the two phages with certainty and is more like the phage-organism relationship found (on a wider scale) by Morison (7) with cholera phages. Organism K is sensitive to *k* and *b* phages. Strain K/*k* is resistant to *b*, but strain K/*b* is still sensitive to *k*.

It is evident from a study of the interrelationships of even the few phages and strains of lactic streptococci so far examined in detail that no simple hypothesis will cover the phenomena. It would appear to be necessary to postulate complex antigenic "mosaic" structures not only for the organisms but also for the phages. This, however, would lead to the elaboration of a hypothesis too "top-heavy" to serve any useful purpose in a further examination of the phenomena. More facts need to be gathered in connexion with phage action on lactic streptococci before we can be sure that we have a reasonable "cross-section" of the phenomenon for critical examination.

SUMMARY

The methods used for isolation and purification of bacteriophages active against lactic streptococci are described. An account is given of the relationships between nine apparently distinct phage races and eleven strains of lactic streptococci. The phages show a tendency towards strain specificity but there are some races which attack up to four different strains. Resistant strains usually arise on continued incubation of lysed cultures. Cross-resistance tests, in those cases where two or more phages act on one strain of streptococci, indicate that the relationships between the phages do not follow any simple rule.

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235. THE ENRICHMENT OF *AEROGENES-CLOACAE* TYPES IN MILK HELD AT LOW TEMPERATURES: WITH OBSERVATIONS ON THE RELATIVE RATES OF GROWTH OF *AEROGENES-CLOACAE* AND *B. COLI* TYPES IN MILK AT DIFFERENT TEMPERATURES

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In an investigation of the types of coliform bacteria occurring in milk (Malcolm(1)), it was found that during winter, when cows are confined to byres, the ratio of "typical" *B. coli* (Muir & Ritchie(2)) to other coliform bacteria, e.g. *B. aerogenes*, *B. cloacae*, etc., in milk was 2·4 : 1, whereas in summer, when cows were at pasture, the ratio was 0·7 : 1. Since typical *B. coli* are the predominant types in bovine faeces, it was suggested that this variation was associated with a greater possibility of faecal contamination of the milk when the cows were confined to byres than when they were at pasture. However, it was considered necessary to carry out a further investigation to determine the source of the other coliform (hereinafter referred to as *aerogenes-cloacae*) types in milk, and also the conditions which are responsible for their prevalence during the summer. In this investigation (Malcolm(3)) it was shown that, while the typical *B. coli* were the predominant coliform types in bovine faeces, the *aerogenes-cloacae* types were almost always present, though in considerably smaller numbers. Consequently, it was thought that bovine faeces might constitute an important primary source of the *aerogenes-cloacae* types in milk, for, though they might be only scantily present in faeces, it was possible that these organisms, being relatively resistant, might become enriched in the utensils and elsewhere and in the milk. The present investigation was therefore carried out to find whether the *aerogenes-cloacae* types might not become enriched in milk held at low temperatures, such as are used in dairy practice.

1. Rate of multiplication of pure cultures of coliform organisms in milk held at 17° C.

Seventy-five cultures of various types of coliform organisms (*B. coli*, *B. aerogenes*, *B. cloacae* and *B. oxytocus*) were used in this experiment; thirty-one of these cultures had been obtained from milk, twenty-nine from bovine faeces, six from bovine urine and nine from human sources. Twenty-two of the cultures had been in stock for several years, but the remaining fifty-three were recently isolated. In all instances, before being used in the experiment, the

cultures were subcultured daily at 37° C. for 7 or 8 days on yeastrel milk agar (Wilson(4)) slopes. Then, from a 24 hr. old agar-slope culture, a saline suspension was prepared; a 1/100,000 dilution of the suspension was made in sterile water; and 1 ml. of this dilution was added to 50 ml. of milk, which had been partially or completely sterilized. (Partial sterilization was carried out by a single heating of the milk for over ½ hr. in a Koch's steam sterilizer, followed by immediate cooling to 5° C. Complete sterilization was carried out by the intermittent method. There was no significant variation in the results of the experiments according to the method of sterilization employed.) In the latter part of the investigation the amount of inoculum was reduced, and steps were taken to ensure that in different experiments the milk would be inoculated with approximately the same number of bacteria. Thus the saline suspensions, prepared from the agar-slope cultures, were standardized to contain approximately 300,000,000 organisms per ml. by the opacity method of Brown & Kirwan(5)). Consequently, in these experiments the 1/100,000 dilution con tained roughly 3000 bacteria per ml. and the inoculated milk 60 per ml. However, in all instances the bacterial content of the inoculated milk was determined by the plating method, plates being made in yeastrel milk agar with 1, 0·1 and 0·01 ml. of the milk. At first the 1 and 0·1 ml. plates were prepared in duplicate, and the 0·01 ml. plates in quintuplicate (Malcolm(6)), but later, when the amount of inoculum was reduced and standardized, the 1 ml. plates were made in quintuplicate and the 0·1 ml. plates in duplicate, and no 0·01 ml. plates were used. (The 1 and 0·1 ml. plates were prepared by measuring 1 and 0·1 ml. of the milk into the respective Petri plates; the 0·01 ml. plates, by adding 1 ml. of the milk to 99 ml. sterile water and transferring 1 ml. of this dilution to each plate.) The plates were incubated at 37° C. for 48 hr. and the colonies counted.

The inoculated milk was incubated at 17° C., this being the lowest temperature that could be obtained by the low-temperature incubator in use at the commencement of the experiment. (This temperature corresponds closely with the temperature at which milk of the "Tuberculin Tested" grade and ordinary milk is held and marketed in Scotland in summer, but it is higher than the temperature frequently found in the case of "Certified" milk. In accordance with the Milk (Special Designations) Order (Scotland), 1936, "Tuberculin Tested" milk must be cooled to 60° F. or 15·5° C., and "Certified" milk to 50° F. or 10° C.) After the milk had been incubated for 24 hr., another bacterial count was made. In the earlier part of the investigation dilutions of 1/1000, 1/10,000 and 1/100,000 were prepared to enable duplicate plates for 0·001 ml., quintuplicate plates for 0·0001 ml. and duplicate plates for 0·00001 ml. quantities of the milk to be made, but later when the amount of the inoculum was reduced, dilutions of only 1/100 and 1/1000 were made, quintuplicate plates being prepared for 0·01 ml. of the milk, and duplicate plates for 0·001 ml.

The results of this experiment are given in Table I. The initial and final counts are shown and also the ratios of the latter to the former. It will be seen

Table I. *The rate of multiplication of different types of coliform organisms in pure culture in sterilized or partially sterilized milk held at* 17° C. *for* 24 *hr. The cultures belonging to each subgroup are arranged in ascending values of the ratios of final counts to initial counts*

Type of organisms	Initial count per ml.	Final count per ml.	Ratio of final count to initial count
B. coli subgroup (30 strains)	3,700	70,000	19
	4,800	90,000	19
	79,600	1,960,000	25
	13,900	500,000	36
	14,900	650,000	44
	4,400	200,000	45
	39*	2,300	59
	18,500	1,110,000	60
	59*	4,300	73
	13,700	1,020,000	74
	33*	2,600	79
	6,800	570,000	84
	35*	3,100	89
	1,660	160,000	96
	12,800	1,240,000	97
	11,600	1,150,000	99
	7,000	740,000	106
	41*	4,900	120
	29*	3,500	121
	43*	5,400	126
	59*	8,100	137
	6,600	1,090,000	166
	6,900	1,170,000	170
	29*	5,900	203
	3,800	770,000	203
	30*	7,900	263
	33*	9,000	273
	88*	25,600	291
	28*	8,500	304
	49*	20,900	427
B. cloacae subgroup (11 strains)	2,400	1,555,000	648
	100*	66,000	660
	50*	37,900	758
	36*	29,000	806
	66*	54,000	818
	3,050	2,970,000	974
	56*	63,000	1,125
	61*	71,000	1,164
	46*	95,000	2,065
	1,050	3,530,000	3,362
	7,600	47,610,000	6,264
B. oxytocus subgroup (20 strains)	14*	11,600	829
	58*	52,300	902
	19*	19,000	1,000
	1,400	1,470,000	1,050
	40*	44,000	1,100
	47*	53,000	1,128
	131*	161,000	1,229
	58*	72,000	1,241
	33*	42,000	1,273
	77*	105,700	1,373
	36*	50,000	1,389
	60*	86,000	1,433
	47*	89,000	1,894
	55*	105,000	1,909
	74*	168,500	2,277
	81*	205,500	2,537
	850	3,150,000	3,706
	57*	215,000	3,772
	450	2,105,000	4,678
	800	6,880,000	8,600

Table I. (*continued*)

Type of organisms	Initial count per ml.	Final count per ml.	Ratio of final count to initial count
B. aerogenes subgroup (14 strains)	3,700	2,135,000	577
	56*	35,300	630
	7,900	6,130,000	776
	18*	15,400	856
	119*	129,000	1,084
	4,250	4,665,000	1,090
	43*	67,000	1,558
	47*	99,000	2,106
	65*	140,000	2,154
	1,400	3,390,000	2,421
	46*	145,000	3,152
	7,200	23,240,000	3,228
	8,300	26,830,000	3,233
	3,800	14,840,000	3,905

* Employing standardized suspensions. See text.

from these ratios that in all instances the *B. aerogenes*, *B. cloacae* and *B. oxytocus* cultures have multiplied more rapidly than the *B. coli*, and in many cases at much higher rates. But considerable variation in the rate of multiplication is shown by different strains of the same coliform subgroup. Thus the ratio of the final to the initial count varies with the *B. coli* cultures from 19 to 427; with the *B. cloacae*, from 648 to 6264; with the *B. oxytocus* from 829 to 8600; and with the *B. aerogenes* from 577 to 3905. No significant difference was observed in the rate of multiplication according to whether the culture had been in stock for several years or had been recently isolated.

There was a possibility that the numbers of coliform bacteria in the milk, as estimated by the plating method, might be affected by the occurrence of the organisms in clusters, instead of separately, in the liquid. This, however, did not appear to be the case with the initial counts, because, as will be seen from Table I, when standardized suspensions were used for inoculating the milk, there was a fairly close agreement between the initial counts of the various bottles of inoculated milk, and these counts also agreed closely with the estimate based on the count to which the primary suspensions were standardized by the opacity method. Nevertheless, there was a possibility that clumping of the bacteria might occur in the milk during the period of incubation, i.e. while the organisms were growing and multiplying. To determine whether this was the case a further experiment was carried out employing thirty-two of the cultures used in the previous experiment, fifteen of these cultures being of *B. coli* types and seventeen of *aerogenes-cloacae*.

By the procedure already described, standardized suspensions containing approximately 300,000,000 organisms per ml. were prepared from 24 hr. old agar-slope cultures. Stained smear preparations were made from these suspensions and examined microscopically to confirm the fact that the organisms did not occur in clusters. The suspensions were then diluted 100,000 times and 1 ml. amounts transferred to bottles containing 50 ml. of partially sterilized milk, as in the previous experiment. As the inoculated milk contained roughly

sixty bacteria per ml., it was not possible to determine by microscopic examination whether clumping of the organisms had occurred on the addition of the inoculum to the milk, but, as previously mentioned, the fact that the plate counts agreed fairly closely with this estimate showed that clumping had not taken place.

The bottles of inoculated milk were incubated at 17° C. The period of incubation was extended to 3 days to allow the bacteria to multiply to such an extent that, on microscopic examination of stained smears from the milk, a considerable number of organisms would occur in each microscopic field. There was the additional advantage that should there be any tendency of the organisms to form clusters, the effect of this would be much greater after 72 hr. incubation than after only 24 hr. Stained smear preparations of the milk were made after 48 and 72 hr. incubation, and these were examined microscopically to determine whether the organisms occurred in clusters or not.

Microscopic examination of the primary suspensions and of the milk cultures after 48 and 72 hr. incubation showed that in all instances there was no tendency to clumping. The organisms occurred in the smears either singly or in pairs. Clusters were very rarely observed, and these in all cases were small in size. Accordingly, little or no importance can be attached to the effect of clumping on the coliform counts obtained in the previous experiment.

The marked bacteriostatic action of fresh "raw" milk on certain organisms is destroyed by boiling. Consequently, there was a possibility that in the first experiment the rates of multiplication of the various coliform organisms might have been different if fresh unheated milk had been used instead of milk heated to 100° C. to effect sterilization. The experiment was, therefore, repeated with fresh raw milk. A cow proved to be free from mastitis was selected from which to obtain milk of a low bacterial content. The cow's udder and teats and the worker's hands were carefully washed with antiseptic. A considerable quantity of milk was discarded from each teat, then part of the remainder drawn directly into large sterile bottles. The milk was cooled immediately by immersing the bottles in cold water, and thereafter brought to the laboratory and placed in a refrigerator. Then 4 hr. after milking, the specimen was thoroughly mixed and distributed in 40 ml. amounts in sterile glass-stoppered bottles. The different lots of milk were then inoculated, as in the first experiment, from standardized suspensions of pure cultures of coliform bacteria. In this case, however, owing to the fact that other organisms might be present in the milk, a heavier inoculation was made to ensure that the coliform organisms would preponderate. Thus each bottle of milk was inoculated with 1 ml. of the 1/10,000 dilution of the primary suspension of the organism instead of 1 ml. of the 1/100,000 dilution. Twenty-nine cultures of different coliform organisms were used in this experiment, thirteen of these cultures were of *B. coli* types and sixteen of *aerogenes-cloacae*. As control, two bottles of milk were not inoculated. The bacterial numbers present in the inoculated milks and in the

controls were then determined by the plating method, triplicate plates being made with 0·1 and 0·01 ml. quantities of the inoculated milks and with 1, 0·1 and 0·01 ml. quantities of the controls. The counting was carried out after the plates had been incubated at 37° C. for 48 hr. The number of coliform organisms with which each bottle of milk had been inoculated was then calculated by subtracting the average bacterial content of the two controls (282 organisms per ml.) from the bacterial content of the inoculated milk.

The bottles of inoculated milk and of the controls were kept at 17° C. for 24 hr. and then the number of organisms in each again determined. In this case plates were made with 0·001, 0·0001 and 0·00001 ml. quantities of the inoculated milks and 0·01, 0·001 and 0·0001 ml. quantities of the controls. The plates were incubated at 37° C. for 48 hr. and the counts made. The final coliform count of each bottle of inoculated milk was then calculated by subtracting the average bacterial content of the two controls after incubation (2800 organisms per ml.) from the final bacterial content of the inoculated milk.

The results of this experiment (see Table II) showed that the raw milk had

Table II. *The rate of multiplication of different types of coliform organisms in pure culture in a specimen of raw milk held at* 17° C. *for* 24 *hr. The cultures belonging to each subgroup are arranged in ascending values of the ratios of final counts to initial counts*

Type of organisms	Initial count per ml.	Final count per ml.	Ratio of final count to initial count
. coli subgroup (13 strains)	1,868	700	0·5
	2,618	1,200	0·5
	2,208	2,700	1
	2,638	10,200	4
	2,908	16,200	6
	3,518	22,700	6
	6,118	34,200	6
	4,118	51,200	12
	1,328	58,700	44
	3,418	171,700	50
	2,318	122,200	53
	3,018	198,200	66
	2,218	169,200	76
B. cloacae subgroup	4,018	80,200	20
(3 strains)	2,198	502,200	228
	1,958	3,997,200	2,042
B. oxytocus subgroup	10,218	18,200	2
(7 strains)	2,418	827,200	342
	2,618	992,200	379
	2,318	2,052,200	885
	2,148	2,762,200	1,286
	2,338	3,794,200	1,623
	2,118	4,197,200	1,982
B. aerogenes subgroup	4,218	4,700	1
(6 strains)	1,788	627,200	351
	2,818	2,237,200	794
	3,448	3,137,200	910
	2,818	4,997,200	1,773
	3,118	7,597,200	2,437
Control without addition of any culture	282	2,800	10

The initial and final counts of each coliform type were obtained by subtracting the initial and final counts respectively of the control from the initial and final counts of each milk culture.

a marked bacteriostatic action on certain strains of both *B. coli* and *aerogenes-cloacae* types, the *aerogenes-cloacae* types being generally affected to a much less extent than the *B. coli*. (This is what one would expect, taking into account the fact that the *aerogenes-cloacae* types, unlike the *B. coli*, are frequently encapsulated.) The rates of multiplication of the various organisms were on the whole much lower with the raw milk than with the sterilized. Nevertheless, in the case of the raw milk, as in the case of the sterilized, the *aerogenes-cloacae* types multiplied as a rule much more rapidly than the *B. coli*. Thus the findings of the first experiment, i.e. *aerogenes-cloacae* types multiply much more rapidly than *B. coli* in milk held at 17° C., hold good, no matter whether raw or heat sterilized milk is used.

In carrying out the experiment with raw milk, the inoculated specimens after 72 hr. incubation at 17° C. were examined microscopically by the procedure already described, to determine whether there was a tendency for the organisms to occur in clusters. It was found that even after this prolonged period of incubation there was, with the exception of three strains of organisms, little or no tendency to clumping, the cells occurring for the most part either singly or in pairs. Clusters were occasionally observed, but these consisted in many instances of cocci derived from the original flora of the milk. Breed & Stocking(7) obtained similar results with samples of raw milk inoculated with coliform organisms.

The investigation was then continued to determine whether similar results to those of the foregoing experiments would be obtained if mixed cultures, each consisting of a *B. coli* type and an *aerogenes-cloacae* type, were grown at low temperatures in milk.

2. The rate of multiplication of *B. coli*, *B. cloacae*, *B. oxytocus* and *B. aerogenes* types, when growing in mixed cultures of coliform types in milk held at 17° C.

The mixed culture in all instances consisted of a *B. coli* and an *aerogenes-cloacae* type. As in the previous experiments, saline suspensions of pure cultures of the organisms were prepared and standardized by the opacity method to contain approximately 300,000,000 organisms per ml. Then 1/100,000 dilutions of the suspensions were made in sterile water, and the mixed culture was prepared by adding 0·5 ml. of the *B. coli* suspension and 0·5 ml. of the *aerogenes-cloacae* suspension to 50 ml. of sterile milk. The bacterial content of the milk was now estimated, the number of each type of organism present being determined by a method, which was based on the fact that cultures of *aerogenes-cloacae* types in many instances do not grow at 45° C. (see note, p. 424). Only those *aerogenes-cloacae* cultures were used which failed to grow at this temperature. The procedure adopted was briefly as follows: dilutions of 1/10 and 1/100 were prepared from the milk, and plates were made in quadruplicate for 1, 1/10 and 1/100 ml. amounts of the milk. Of each set of quadruplicate plates, two were incubated at 37° C. and two at 45° C. The colonies were counted at

the end of 48 hr. incubation. The number of colonies on the plates which had been incubated at 45° C. indicated the number of *B. coli* organisms present in the milk, and the number of colonies on the plates incubated at 37° C. indicated the number of organisms of both *B. coli* and *aerogenes-cloacae* types. By subtracting the first count from the second, the number of *aerogenes-cloacae* organisms present in the milk was obtained. The ratio of the number of bacteria of *aerogenes-cloacae* type to the number of *B. coli* type was now estimated.

The inoculated milk was incubated at 17° C. for 24 hr., and then a second count was made of each type of organism present, the procedure being the same as before, but dilutions of 1/100, 1/1000, 1/10,000 and 1/100,000 being employed. The ratio of the number of *aerogenes-cloacae* organisms to the number of *B. coli* was estimated. Then by comparing this ratio with the corresponding ratio for the initial counts, the relative rates of multiplication of the two types of organisms present in the milk were determined.

The results of this experiment are shown in Table III, only the ratio expressing the rate of multiplication of the *aerogenes-cloacae* type relative to

Table III. *The relative rates of multiplication of* aerogenes-cloacae *types to* B. coli, *when growing in mixed culture in milk at* 17° C. *for* 24 *hr.*

Exp.	Ratio of multiplication rates: *aerogenes-cloacae* to *B. coli*	Exp.	Ratio of multiplication rates: *aerogenes-cloacae* to *B. coli*
1	5·3	21	0·6
2	31·8	22	11·0
3	6·3	23	3·7
4	33·4	24	2·0
5	1·4	25	12·5
6	0·4	26	5·2
7	19·4	27	3·0
8	8·3	28	9·0
9	7·8	29	0·3
10	5·0	30	25·6
11	24·4	31	14·1
12	7·7	32	3·1
13	11·0	33	6·3
14	52·5	34	4·0
15	5·0	35	0·1
16	4·9	36	0·3
17	2·8	37	14·1
18	0·2	38	4·0
19	90·7	39	21·2
20	7·4	40	17·4

the *B. coli* type being given. It will be seen from the table that with forty mixed cultures of an *aerogenes-cloacae* with a *B. coli* type, the former in thirty-four instances multiplied more rapidly than the latter, while in only six instances did the reverse occur. However, there were great variations; thus the ratios expressing the relative rates of multiplication of the *aerogenes-cloacae* and *B. coli* types ranged from 90·7 to 0·1, although the majority (twenty-eight out of forty) were between 25·6 and 2·8.

3. The influence of temperature on the relative rates of multiplication of cultures of *B. coli* and *aerogenes-cloacae* types, when growing in mixed cultures of coliform types in milk

It was evident from the results obtained both in the second and in the first section of the investigation that the *aerogenes-cloacae* types of bacteria generally multiplied much more rapidly in milk at 17° C. than did the *B. coli* types. While the second part of the investigation was in progress, the question arose whether temperature had any marked influence on the results. Accordingly, in twenty-one instances the milk, after being inoculated with the cultures of the two types of coliform bacteria and the plates for the estimation of the bacterial numbers had been made, was divided into four portions. These were incubated for 24 hr. respectively at 17, 22, 30 and 37° C. The number of *B. coli* and the number of *aerogenes-cloacae* in each portion of the milk were then determined by the method already described; and the ratio of the *aerogenes-cloacae* count to the *B. coli* and the ratio expressing the rate of multiplication of the *aerogenes-cloacae* to that of the *B. coli* were estimated as shown in Section 2.

Table IV. *The influence of temperature on the relative rates of multiplication of* aerogenes-cloacae *and* B. coli *types, when growing in mixed culture in milk for 24 hr. Each figure indicates the ratio of the multiplication rate of the* aerogenes-cloacae *type to the multiplication rate of the* B. coli

	Temperature of incubation, ° C.			
Exp.	17	22	30	37
21	0·6	9·2	0·9	5·0
22	11·0	13·3	—	11·7
23	3·7	43·1	0·3	0·4
24	2·0	77·0	1·7	1·3
25	12·5	7·0	0·3	0·2
26	5·2	20·7	1·3	0·3
27	3·0	1·5	0·3	0·3
28	9·0	34·8	0·2	0·1
29	0·3	0·2	0·7	0·1
30	25·6	21·5	1·3	0·4
31	14·1	23·1	0·1	0·3
32	3·1	1·5	3·8	0·3
33	6·3	5·0	0·3	0·7
34	4·0	7·4	0·5	0·2
35	0·1	0·7	0·2	0·1
36	0·3	0·4	0·1	0·4
37	14·1	8·9	0·1	0·3
38	4·0	5·4	0·2	0·4
39	21·2	19·8	0·3	0·4
40	17·4	25·5	0·2	0·1
41	—	46·7	7·2	3·8

The results of this experiment are given in Table IV, only the ratios expressing the relative rates of multiplication of the *aerogenes-cloacae* and *B. coli* types at the different temperatures being shown. It will be seen that at 17° C. the *aerogenes-cloacae* types multiplied more rapidly than the *B. coli* in sixteen instances out of twenty; at 22° C., they did so in eighteen cases out of twenty-one; at 30° C., in only five instances out of twenty; and at 37° C., in only four

out of twenty-one. Accordingly, temperature has a marked effect on the relative rates of multiplication of the *aerogenes-cloacae* and *B. coli* types in milk. There may be considerable variation between different cultures of the same type with regard to the rate of multiplication at a given temperature, but the *aerogenes-cloacae* types generally multiply more rapidly than the *B. coli* in milk held at 17 and 22° C., while the *B. coli* types in most instances multiply more rapidly than the *aerogenes-cloacae* in milk held at 30 and 37° C.

4. THE COLIFORM FLORA OF RAW MILK, WHICH HAS BEEN INOCULATED WITH BOVINE FAECES, AND HELD FOR 36 HR. AT 17° C.

This experiment was carried out to determine whether enrichment of *aerogenes-cloacae* strains occurs when bovine faeces containing them are inoculated into milk and the culture kept for 36 hr. at 17° C. The milk used in the experiment was unheated fresh milk of the Scottish "Certified" grade from farms known to produce clean milk of low bacterial count. On its arrival in the laboratory it was subjected to the coliform test, 1 ml. quantities being inoculated in triplicate into MacConkey's bouillon. Although the results of this test could not be determined until the bouillon cultures had been incubated for 2 days, the milk was used immediately for experimental purposes, the results being discarded when the milk was shown to contain coliform bacteria.

In carrying out the experiment, the milk was added in 50 ml. amounts to sterile bottles. The bottles were then inoculated with different specimens of bovine faeces, which were from 1 to 3 days old. In the case of the first six faecal specimens two loopfuls of the faeces were used as the inoculum in each instance. But with the last six specimens the quantity of the inoculum was reduced and various amounts were used. Thus the practice was to prepare a fairly heavy suspension of each of these faecal specimens in sterile water and to inoculate separate bottles of milk with 1, 0·5 and 0·25 ml. of the suspension. The bottles of inoculated milk were incubated in all instances at 17° C. for 36 hr. Then three plates of MacConkey's agar were stroked in series from each bottle and incubated at 37° C. for 2 days. Over thirty colonies were picked off each set of triplicate plates and transferred to MacConkey's bouillon. Accordingly, over thirty bouillon cultures were obtained from each bottle of inoculated milk, i.e. over thirty bouillon cultures from each of the first six faecal specimens and over ninety from each of the last six specimens. The bouillon cultures which produced acid and gas were replated in MacConkey's agar to ensure their purity. Then a subculture in MacConkey's bouillon was made from each plate. If acid and gas were produced the organisms were examined microscopically, after staining by Gram's method, and identified by their Voges-Proskauer, Koser, inositol and indole reactions (Malcolm(8)).

The results of this experiment are given in Table V. It will be seen that in fourteen instances out of twenty-four, strains of the *aerogenes-cloacae* and intermediate types together constituted the greater proportion of the flora of the milk. In twelve cases the *aerogenes-cloacae* strains by themselves were in

excess of the *B. coli*, while in twelve other cases the reverse occurred. With four of the six faecal specimens, when 0·25 ml. of the faecal suspension was used as the inoculum, *aerogenes-cloacae* strains were obtained from the milk, and in three of these instances they were the predominant coliform strains. Accordingly, although *aerogenes-cloacae* types occur in bovine faeces only in comparatively small numbers, they become enriched in milk inoculated with the faeces and held at 17° C. Consequently, they are frequently the predominant coliform types in such milk.

Table V. *The coliform flora of milk which has been inoculated with bovine faeces and held for* 36 *hr. at* 17° C.

	Number of cultures isolated from the milk		
Faecal specimen	*B. coli* types	Intermediate types	*Aerogenes-cloacae* types (*B. aerogenes*, *B. cloacae*, *B. oxytocus* and *B. Friedländer*)
1	—	—	26
2	1	5	23
3	6	4	3
4	1	3	—
5	—	—	2
6	—	2	7
7*a*	28	—	—
b	14	—	—
c	10	1	—
8*a*	33	—	—
b	30	—	1
c	30	2	—
9*a*	—	5	9
b	2	3	10
c	6	1	18
10*a*	26	—	—*
b	7	—	—
c	8	—	—*
11*a*	—	1	13
b	4	1	6
c	2	—	8
12*a*	5	3	1
b	2	—	7
c	4	—	12

* Cultures of *aerogenes-cloacae* types were obtained on re-examination of colonies on original plates.

Inoculum consisted in "*a*" of 1 ml. of faecal suspension; in "*b*" of 0·5 ml.; and in "*c*" of 0·25 ml.

Discussion

The significance of these results with regard to the incidence of various coliform types in unpasteurized market milk requires to be considered and in this connexion the following facts are of importance.

1. Previous work (Malcolm(1,3)) has shown that the most prevalent types of coliform bacteria in unpasteurized market milk are *B. coli communior* and *B. coli communis* Escherich and these are also the most prevalent coliform types in bovine faeces. Further, there is a very high correlation between the relative numbers of *B. coli communior* and *B. coli communis* in milk and in bovine faeces, *B. coli communior* occurring in each case with greater frequency

than *B. coli communis*. On the other hand, these coliform types have been found by numerous investigators to be of comparatively rare occurrence on fodder, grain and in soil, in the absence of recent faecal contamination.

2. It has also been shown that the relative frequency of strains of different coliform subgroups in unpasteurized market milk varies widely according to winter and summer conditions. During winter 71·0% of the strains obtained from specimens of such milk were typical *B. coli*, 16·1% were *aerogenes-cloacae* types and 12·9% were intermediate types, whereas during summer 40·4% of the strains were typical *B. coli*, 32·2% were *aerogenes-cloacae* types and 27·4% were intermediate types.

3. In winter there is a much greater possibility of gross faecal contamination of the milk than in summer. This is due to the fact that in Scotland during winter the cows are confined almost entirely to byres, being allowed out during the day to the fields only for a brief period for exercise. In summer they are at pasture and are in the byres only at milking time.

4. While the *aerogenes-cloacae* types may occur naturally on vegetation, their incidence in milk is highest in summer. At this season the cows are at pasture and brought into the byres only at milking time. Consequently, they are not provided with fodder and bedding and there is little or no possibility of the milk being contaminated with hay, ensilage and straw, or dust from these materials. It is possible that contamination from the coats of the cows may occur in summer from contact with pasture plants, but as the animals move freely about the fields, they themselves in many, if not in most, instances may be responsible for the presence of coliform bacteria on the plants. In any case, Wilson(4) found that *aerogenes-cloacae* types were comparatively seldom present on what he termed the "soil-contaminated materials"—straw, hay, grass, decaying leaves, water and swedes. He frequently obtained them from cereal grains, meals and feeding cakes, but there is little or no possibility of milk being contaminated from these sources in summer, as at this season the cows, being at pasture, receive only very small quantities, if any, of such foods. On the other hand, the coats of the cows may be contaminated with mud in summer. This, however, is more likely to happen in winter than in summer, as in winter the fields, roads and gateways where the cows go for exercise, are generally in a wetter and muddier condition. In any case, such mud is as a rule heavily contaminated with faeces, and the coliform organisms present will in all likelihood be of faecal origin. In this connexion it must be remembered that Houston(9), Burke-Gaffney(10), Bardsley(11) and others, have found that coliform organisms are frequently absent from soils which are free or relatively free from faecal contamination. Moreover, Bardsley(11) found that where coliform organisms occur in such soils in Britain they are largely of *B. coli* and intermediate types (31·0% *B. coli* and 66·4% intermediate), *aerogenes* types being comparatively rare. Wilson(4), in discussing the comparative absence of *aerogenes-cloacae* types from soil-contaminated materials such as straw, hay, grass, water and swedes, states

that this "to some extent confirms Bardsley's observations on the infrequency of *aerogenes-cloacae* organisms in the soil of this country". Although dust is more prevalent in summer than in winter, it arises chiefly from roads, especially untarred farm roads, and not from fields carrying their summer crops. Such road dust is almost certain to contain particles of faeces. Wilson(4) suggests that milk may be contaminated with *aerogenes-cloacae* types in summer from the dust from feeding substances, but, as previously stated, in summer such foods are either not given at all or only to a very limited extent.

5. It has been found in the present investigation (*a*) that *aerogenes-cloacae* types as a rule multiply much more rapidly in milk at low temperatures, 17–22° C., than *B. coli* types, this being the case whether raw or partially sterilized milk is used, and (*b*) that the coliform flora of milk which has been inoculated with bovine faeces and held for 36 hr. at 17° C. frequently consists chiefly of *aerogenes-cloacae* types. These results are in agreement with those of other investigators. Thus, Rogers *et al.*(12) quote Ayers as finding that when sterile milk was inoculated with bovine faeces and incubated at 20° C. the *aerogenes* types predominated at the time of curdling, and they found thatthis also holds good for human faeces. Hammer(13) states that the "*Aerobacter* type" can grow in milk at a lower temperature than the "*Escherichia* type", so that it is of more importance from the standpoint of development in milk under ordinary conditions.[1] From these facts the inference may be drawn that if milk which is contaminated with faecal matter is kept at temperatures of from 17 to 22° C., it may ultimately contain large numbers of *aerogenes-cloacae* types, even though these organisms occurred scantily in the faeces. Such a state of affairs is liable to arise in summer because, as previously mentioned, 17° C. (63° F.) is a common temperature for farm milk at that period. On the other hand, milk is generally kept in winter at such low temperatures, e.g. 5° C. (41° F.) to 13° C. (55° F.), i.e. at about 10° C. (50° F.), that there is little or no growth and proliferation even of *aerogenes-cloacae* types. (Ayers & Clemmer(14) and Finkelstein(15) found that three was little or no growth of any coliform types in milk at or below 10° C.) Consequently, the relative incidence of the different coliform types in the milk will tend to remain unaltered, and will correspond fairly closely with that of the different types in the faeces with which it was contaminated.

6. *B. lactis aerogenes* and *B. oxytocus*, i.e. the encapsulated types of coliform bacteria, are much more resistant to adverse conditions generally than the non-capsulated types, e.g. the typical *B. coli*. Thus Clemesha(16), Rogers(17), Winslow & Cohen(18), Gray(19), Burke-Gaffney(20), Platt(21), and others, have found that *B. aerogenes* has greater powers of resistance and can generally survive longer than *B. coli* in water. Accordingly, if milk utensils which have

[1] Wilson(4) found that when eight mixed cultures of *B. coli* and *B. aerogenes* strains were grown for 24 hr. in milk at 22° C., the *B. coli* in all instances multiplied more rapidly than the *B. aerogenes*. But he makes no claim that general conclusions can be drawn from his experiments owing to the small number of strains examined.

become contaminated with coliform organisms as a result of faecal contamination of the milk or of the utensils themselves, are not properly washed and sterilized, the *B. aerogenes* types will very possibly survive in them to a greater extent than the *B. coli* types. This is supported by the results of an investigation carried out by Prof. R. H. Leitch of the West of Scotland Agricultural College. In a personal communication he stated that he found that milk is frequently infected with coliform bacteria and especially *aerogenes* types from the utensils.

In view of these facts, it seems reasonable to suppose that not only the typical *B. coli*, but also the *aerogenes-cloacae* types present in milk, are derived to a considerable extent from bovine faeces. The relatively higher incidence of the latter in summer milk can be explained in terms of the foregoing findings as being due to a combination of factors. Their greater hardiness will allow them to survive in improperly sterilized utensils, while the temperature of the environment in the summer months will be almost optimal for their multiplication both in the utensils, e.g. the cans, strainer and cooler, and in the milk itself. Thus given faecal contamination of the milk, for which there is abundant evidence, even the small numbers of *aerogenes* types present in the faeces will, under these suitable conditions for growth, form a large proportion of the coliform types in milk during the summer months. They will be, however, relatively rare in winter when conditions are more suitable for gross faecal contamination of the milk, and when, owing to the low temperature of the environment, there will be comparatively little growth even of these types of coliform organisms in the utensils and in the milk.

SUMMARY

1. Seventy-five pure cultures of various types of coliform bacteria were grown in sterilized or partially sterilized milk for 24 hr. at 17° C. The cultures of *B. aerogenes*, *B. oxytocus* and *B. cloacae* types as a rule multiplied much more rapidly than those of the *B. coli* types. Similar results were obtained when twenty-nine cultures of various coliform types were grown in raw milk for 24 hr. at 17° C. There was generally little or no tendency to clumping of the organisms in the milk, even after 3 days' incubation at 17° C.

2. Forty mixed cultures, each consisting of an *aerogenes-cloacae* type and a *B. coli*, were grown in milk for 24 hr. at 17° C. In thirty-four instances the *aerogenes-cloacae* type multiplied more rapidly than the *B. coli*.

3. Twenty-one of these mixed cultures were also grown in milk for 24 hr. at 22, 30 and 37° C. At 22, as at 17° C., the *aerogenes-cloacae* type multiplied as a rule more rapidly than the *B. coli*, while at 30 and 37° C. the reverse was the case.

4. Twelve specimens of bovine faeces were inoculated into raw milk and the cultures kept at 17° C. for 36 hr. *Aerogenes-cloacae* types occurring in the faeces became enriched in the milk, the coliform flora of the milk at the end of the incubation period frequently consisting chiefly of these types.

5. The greater incidence of *aerogenes-cloacae* types in summer milk in Scotland as compared with winter milk, found in a previous investigation, may be explained by the fact that when milk is contaminated directly or indirectly with faeces, these types become enriched at the temperatures of holding commonly employed in summer. Such enrichment does not occur in winter, the holding temperature of the milk being as a rule so low that there is little or no proliferation of any coliform types. Consequently, the relative proportions of the various coliform types in winter milk tend to remain similar to those in the faeces.

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NOTE

All the cultures of *B. coli* type used in the investigation grew well in bouillon at 45° C. There was, however, a possibility that the agar plate counts of such cultures might be less, if the plates were incubated at 45 than at 37° C. An experiment was therefore carried out to determine whether this was the case. Bouillon cultures of nine different *B. coli* strains, which had been subcultured daily for several days, were plated out on yeastrel milk agar. Six parallel plates were made for each of the following quantities of the cultures: 0·00001, 0·000001 and 0·0000001 ml. The plates were incubated for 2 days, three of each set of parallel plates being kept at 37° C. and the remaining three at 45° C. The counts were then made. The following table shows the bacterial content per ml. of each culture as determined by plating at 37 and at 45° C.

Table VI

Strain	Bacterial content per ml. of bouillon culture when plates incubated at 37° C.	Bacterial content per ml. of bouillon culture when plates incubated at 45° C.
1	270,000,000	280,000,000
2	160,000,000	150,000,000
3	300,000,000	420,000,000
4	205,000,000	300,000,000
5	140,000,000	150,000,000
6	269,000,000	423,000,000
7	390,000,000	380,000,000
8	410,000,000	360,000,000
9	570,000,000	450,000,000

It will be seen from the table that in most instances there was a close correspondence between the counts for each strain. Accordingly, with *B. coli* strains the accuracy of the plate count is not affected by employing an incubation temperature of 45 instead of 37° C. On the other hand, it was found that fourteen out of fifty-five *aerogenes-cloacae* strains grew in bouillon at 45° C. Consequently, this method of making a differential count of *B. coli* and

aerogenes-cloacae types can only be employed provided such atypical *aerogenes-cloacae* strains are absent. The fourteen *aerogenes-cloacae* strains which grew at 45° C. were therefore not used in investigating the relative rates of growth of *B. coli* and *aerogenes-cloacae* types in milk.

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236. THE BACTERIAL FLORA OF NEW ZEALAND CHEDDAR CHEESE

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It has been clearly established that the flavour of Cheddar cheese is the result of bacterial action. Consequently, it is of importance to relate the types of organisms present to the effects which they produce in the cheese. Many papers on the bacterial flora of Cheddar cheese have been published during the last 30 years, but, unfortunately, little detailed information from the standpoint of modern taxonomy is available on the groups of lactic acid bacteria present other than the lactic streptococci associated with the early stages of ripening.

Harrison & Connell(1) in their studies on the rate of ripening of Cheddar cheese at different temperatures found that lactobacilli dominated the flora during the later ripening stages. Harding & Prucha(2) showed that organisms of the *Streptococcus lactis* group were present in large numbers in green Cheddar cheese, and they considered that the major ripening changes were brought about by this group. Hastings *et al.*(3) demonstrated the replacement of the lactic streptococci by the *Bacillus bulgaricus* group and certain types of cocci. In the course of their investigations of cheese flavour Evans *et al.*(4) isolated approximately 1000 strains of lactic acid bacteria from thirty-seven raw-milk cheeses. They found that the organisms constantly present in Cheddar cheese in sufficiently large numbers to function in the ripening process were included in the four groups, *Bacterium lactis acidi*, *Bacterium casei*, *Streptococcus* and *Micrococcus*. These four groups were subdivided on the basis of their reactions with five different sugars. The authors concluded that the flora of raw-milk cheese was variable, consisting of all varieties of the four groups, but that the flora of pasteurized-milk cheese, except for organisms of the *Bacterium casei* group, was dependent on the flora of the starter. *B. casei* was considered to be responsible for the pungent taste which develops in old cheese; inoculation of the cheese-milk, however, with strains of this group proved somewhat detrimental to the quality of the cheese. On the other hand, the addition of certain strains of streptococci in admixture with *B. lactis acidi* appeared to have a beneficial effect. Later, Evans(5) made a study of the streptococci in cheese and reported that the flavour of cheese was improved by the addition of *Streptococcus* X and *Streptococcus kefir* to the cheese-milk. Hucker(6) showed that the flora of high-grade commercial Cheddar cheese consisted mainly of lactic acid bacteria (*Str. lactis* and various lactobacilli), whereas poor quality cheese contained large numbers of sporeformers, Gram-negative rods and, occasionally, organisms of other groups. Kelly(7) investigated the characteristics of the lactic streptococci associated with the early stages of

cheese-ripening, and he came to the conclusion that the main function of these organisms was the development of acid. Allen & Knowles(8) made bacterial examinations of a number of small cheeses manufactured from raw milk of low bacterial count with the use of two starters of different vitality. They found that the flora for at least the first 19–20 weeks consisted mainly of lactic streptococci. In the cheese made with the vigorous starter no lactobacilli appeared. Lactobacilli were isolated from the cheese made with the slow starter during the later ripening stages; most of the strains were found to produce acid very slowly in litmus milk at 37° C.

Davis(9) classified the lactic acid bacteria isolated at regular intervals from a number of cheeses made from Shorthorn milk of English certified standard. Only four species were found—*Str. lactis*, *Str. cremoris*, *Sbm. plantarum* and *Sbm. casei*. *Str. lactis* and *Str. cremoris* were equally viable during the first month of ripening, after which *Sbm. plantarum* and *Sbm. casei* were isolated, the former bacillus being found only when the cheeses were 1–5 months old, the flora consisting entirely of *Sbm. casei* after that time. The general vigour of both *Sbm. casei* and *Sbm. plantarum* decreased with increasing age of the cheese. *Sbm. plantarum* appeared to be affected very quickly, "weakened" strains of this species being isolated, even from young cheese, much more frequently than normally vigorous strains.

PRESENT INVESTIGATION

(1) *Scope.* In New Zealand cheese-making practice the milk is usually flash-pasteurized at a temperature in the vicinity of 155–165° F., and as a result the bacterial content of the milk in the vats is comparatively low. A starter—either a single strain of *Str. cremoris* or a mixture of strains of lactic streptococci—is invariably used. A significant feature of the ripening process is the early displacement of the lactic streptococci by lactobacilli. When clean milk (e.g. plate count after pasteurization, < 2000) is employed, as, for instance, in the Dairy Research Institute factory, lactobacilli usually make their appearance approximately a fortnight after manufacture, but in commercial cheese these organisms dominate the flora earlier, frequently attaining very high numbers, when the cheese is a few days old.

Recent investigations(10,11,12) have indicated that the group of lactobacilli play a very important part in the ripening of New Zealand cheese. Some interest, therefore, attaches to a study of the various types of these organisms which occur.

The objective of the present investigation was threefold: (*a*) to supplement the meagre information available in the literature on the types of lactobacilli and associated organisms which occur in commercial Cheddar cheese; (*b*) to develop a satisfactory method for the routine identification of these organisms; and (*c*) to attempt to correlate the biochemical characteristics of different strains of lactobacilli with the effects which these organisms produce in cheese.

(2) *Isolation of organisms.* Over a period of 5 years New Zealand Cheddar

cheese was examined and samples were taken from representative cheeses for bacteriological analysis. Care was taken to avoid sampling cheeses with characteristics which occurred infrequently. With the exception of certain of the locally manufactured cheeses, a number of which were 40 lb. in weight, all the cheeses were of export size (80 lb.). There was, therefore, no necessity for taking into account any possible differences between the ripening processes of the various cheeses imposed by differences in size. Cheese varying in age from 1 to 80 weeks were selected from all the important cheese-producing districts in New Zealand.

In the present experiments isolations were made from thirty-five New Zealand Cheddar cheeses and from one English Cheddar cheese. (This cheese was included to illustrate a relationship between the organisms occurring in raw-milk cheese made in different localities; the flora was typical of several English Cheddar cheeses examined.) In order to ensure the isolation of types truly representative of the flora of each cheese, samples of 15 g. or more, taken where possible from several plugs, were emulsified and plates poured with suitable dilutions. The medium most frequently employed was yeast-whey agar (two layers), but tomato-whey agar and dextrose-bean agar were occasionally used. In general, however, little was gained by the use of the two latter media. Twenty adjacent colonies on well-spaced plates poured from each cheese were transferred to yeast-milk (10%) and the purified strains were broadly classified according to the following scheme (Table I):

Table I. *Scheme for broad classification of lactic acid bacteria isolated from cheese* (*incubation temperature in all cases*, 30° C.)

Group	Morphology (milk, 2 days)	No. of days required for coagulation of yeast-milk (10%)	No. of days required for reduction of litmus milk	Amount of carbon dioxide evolved in 14 days (g./l.) (yeast-milk)
1. *Sbm. casei*	Square-ended rods, often granulated, fairly long chains	2	4–8	0·15–0·25
2. *Sbm. plantarum*	Rounded rods, short chains	2–3	>12 Usually 40–∞	0·4 –0·8
3. Organisms intermediate between 1 and 2	Rods, variable	2	6–12	0·4 –0·8
4. Betabacteria	Rods, short chains	6–16	∞	1·0 –3·5
5. Betacocci	Cocci, short chains	2–6	10	2·0 –3·0
6. Other lactic acid bacteria (in particular, streptococci)	Variable	Variable	Variable	<0·8

The above scheme has been employed successfully in this Institute over a long period. In other laboratories it is possible that the use of different milk supplies, etc., may cause the values obtained for the characteristics of any particular group to depart slightly from the figures given for that group in Table I; under these conditions, however, the corresponding characteristics of all the groups will undergo a more or less proportionate small change. Consequently, as the six groups are very well defined, an efficient separation should still be obtained.

BROAD CLASSIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM THIRTY-SIX TYPICAL CHEESES

In Table II particulars are given of the cheeses from which isolations were made, and representative strains from each cheese are classified on the basis of the four characteristics tabulated above. The relative numbers of each type present in the various cheeses are also indicated. As will be seen from the table, the data furnished by the four simple determinations is often sufficient to indicate varietal differences. In order to facilitate presentation of data in tables that follow, the representative strains in Table II have, in a few instances, been subdivided on the basis of further tests which will be discussed later.

EXPERIMENTAL

(1) *Judgment of cheese quality.* The quality of the cheese was judged by experienced graders on the Institute Staff and/or experienced graders of the Dairy Division, New Zealand Department of Agriculture. The five cheeses graded "excellent" in Table II were prize-winning exhibits especially selected for excellence of flavour.

(2) *Method of numbering strains.* The number preceding the decimal point represents the cheese from which isolations were made and the figure following the point represents the number of the strain isolated from that cheese, e.g. 2·3 signifies the third strain isolated from cheese no. 2. The letter E indicates a strain isolated by use of an enrichment method (10).

(3) *Determination of the rate of formation of carbon dioxide.* The method has been described in an earlier communication (12). It is essential to provide a comparatively large surface of culture and of absorbing solution. For this reason, Erlenmeyer flasks were employed.

(4) *Temperature of incubation.* Unless otherwise indicated this was 30° C.

Aggregate flora. In tables which follow further data will be given in support of the broad classification shown in Table II.

It will be seen from Table II that the flora of the three raw-milk cheeses (including the English raw-milk cheese) differed appreciably from that of the pasteurized milk cheeses in containing large numbers of cocci. Consequently, it will be convenient to consider these two classes of cheese separately.

The 660 strains of lactic acid bacteria isolated from the thirty-three New Zealand pasteurized-milk cheeses were distributed among the six groups of Table I as follows:

Group	No. of strains	Percentage of total strains
(1) *Sbm. plantarum*	410	62
(2) *Sbm. casei*	106	16
(3) Intermediate (*plantarum-casei*)	78	12
(4) Betabacteria	39	6
(5) Betacocci	20	3
(6) Other L.A.B.	7	1

Table II

Particulars of cheese examined			Organisms studied						
District	Age weeks	Quality	Strain no.	Proportion of the flora*	Morphology (milk, 2 days)	No. days required for (a) Coagulation of yeast-milk	No. days required for (b) Reduction of litmus milk	Amount of carbon dioxide evolved in 14 days g./l.	Broad classification P = *Sbm. plantarum* C = *Sbm. casei* P-C = Intermediate type B = *Betabacterium* Bc = *Betacoccus* — = L.A.B. other than above
Marlborough	12	Excellent	1·1	17	0·7–0·9 × 2–5 μ, short chains	2	∞	0·61	P
			1·2	3	0·8–1·0 × 2–4 μ, very short chains	10	∞	2·97	B
Lower Wairarapa	2–3	Average	2·1	2	0·6–0·9 × 3–5 μ, very short chains	4	∞	0·73	P
			2·2	6	Similar to 2·1, but chains larger	2	18	0·67	P-C
			2·3	12	Similar to 2·2	4	40	0·55	P
Do.	3	Good	3·1	16	0·9–1·1 × 3–5 μ, very short chains	3	∞	0·65	P
			3·2	4	0·5–0·8 × 3–8 μ, granulated, chains	2	7	0·72	P-C
Do.	2–3	Good	4·1	5	0·5–0·7 × 3–8 μ, granulated, long chains	2	5	0·15	C
			4·2	5	0·7–1·0 × 4–8 μ, short chains	2	∞	0·72	P
			4·3	10	0·8–1·0 × 3–6 μ, very short chains	2	∞	0·57	P
Do.	12	Excellent	5·1	16	0·4–0·7 × 2–7 μ, granulated, long chains	2	5	0·18	C
			5·2	4	Similar to 5·1	2	28	0·74	P-C
Do.	12	Good	6·1	4	0·8–1·0 × 3–5 μ, very short chains	16	∞	1·18	B
			6·2	16	0·9–1·1 × 3–5 μ, pairs and very short chains	2	50	0·76	P
Upper Wairarapa	20	Excellent	7·1	6	0·9–1·1 × 3–6 μ, pairs and very short chains	2	∞	0·59	P
			7·2	4	Cocci, 0·7–1·1 μ, long chains	2	40	0·35	—
			7·3	10	Similar to 7·1	2	40	0·79	P
Do.	20	Good	8·1	15	0·4–0·7 × 3–8 μ, granulated	2	6	0·35	P

Do.	20	Good	8·1	15	0·4–0·7 × 3–8 μ, granulated, long chains	2	6	0·25	C
			8·2	5	0·6–0·8 × 2–4 μ, pairs and short chains	9	40	0·72	P
Hawkes' Bay	12	Good	9·1	13	0·6–0·9 × 3–6 μ, pairs and very short chains	2	∞	0·66	P
			9·2	3	0·6–0·9 × 2–5 μ, pairs and very short chains	2	12	0·66	P-C
			9·3	4	Similar to 9·1, but "ragged"	1	∞	0·39	P
Manawatu (D.R.I. Factory)	35	Very poor, discoloured, "off" flavour	10·1	3	0·7–0·9 × 2–5 μ, very short chains	11	∞	2·87	B
			10·2	4	0·7–0·9 × 2–5 μ, short chains	3	∞	0·19	P
			10·3	2	Similar to 10·2	3	∞	0·22	P
			10·4	4	0·7–1·0 × 3–7 μ, short chains	2	∞	0·71	P
			10·5	7	0·8–1·0 × 3–8 μ, short chains	2	∞	0·46	P
Do.	14	Poor, "off" flavour	11·1	11	0·9–1·1 × 3–8 μ, short chains	2	∞	0·48	P
			11·2	9	0·6–0·8 × 2–4 μ, pairs and short chains	2	∞	0·66	P
Do.	12	Very poor, discoloured, open	12·1	13	0·9–1·1 × 3–10 μ, short chains	2	∞	0·50	P
			12·2	7	0·6–0·8 × 2–4 μ, singly and in pairs	3	∞	0·74	P
Do.	12	Average	13·1	16	0·8–1·0 × 3·8 μ, short chains	2	∞	0·50	P
			13·2	4	Similar to 13·1	2	∞	0·68	P
			13·3 E	—	0·8–1·0 × 2–4 μ, very short chains	7	∞	3·52	B
Do.	14	Very poor, "yeasty" flavour	14·1	8	0·8–1·0 × 3–8 μ, short chains	2	∞	0·50	P
			14·2	12	0·8–1·0 × 2–6 μ, very short chains	8	∞	3·50	B
Do.	80	Poor	15·1	10	0·7–1·0 × 2–6 μ, very short chains	16	∞	1·23	B
			15·2	10	0·8–1·1 × 2–4 μ, very short chains	2	50	0·76	P
Do.	35	Poor, discoloured	16·1	8	0·6–0·8 × 2–5 μ, singly and in pairs	3	∞	0·22	P
			16·2	4	0·5–0·8 × 1–3 μ, short chains	6	∞	0·70	P
			16·3	8	0·8–1·0 × 3–8 μ, short chains	2	∞	0·48	P
Do.	24	Excellent	17·1	6	0·7–1·0 × 2–5 μ, short chains	2	∞	0·63	P
			17·2	7	Similar to 17·1	3	∞	0·39	P
			17·3	7	0·5–0·7 × 3–8 μ, granulated, long chains	2	4	0·19	C
			17·4 E	—	0·8–1·0 × 2–4 μ, very short chains	16	∞	1·50	B

* No. of strains out of the 20 isolated from each cheese which appeared to be identical on the criteria of Table I.

Table II (*continued*)

Particulars of cheese examined			Organisms studied						
District	Age weeks	Quality	Strain no.	Proportion of the flora*	Morphology (milk, 2 days)	No. days required for (*a*) Coagulation of yeast-milk	No. days required for (*b*) Reduction of litmus milk	Amount of carbon dioxide evolved in 14 days g./l.	Broad classification P = *Sbm. plantarum* C = *Sbm. casei* P-C = Intermediate type B = *Betabacterium* Bc = *Betacoccus* — = L.A.B. other than above
Manawatu (D.R.I. Factory)	24	Poor, "off" flavour	18·1	12	Cocci, 0·7–1·0 μ, singly and in pairs	3	∞	2·32	Bc
			18·2	8	0·6–1·0 × 3–5 μ, chains	2	∞	0·50	P-C
Do.	14	Average	19·1	17	0·5–0·8 × 3–8 μ, granulated, long chains	2	6	0·21	C
			19·2	3	0·9–1·1 × 3–5 μ, very short chains	3	50	0·78	P
Do.	10	Poor, open	20·1	16	0·8–1·0 × 3–8 μ, short chains	2	∞	0·50	P
			20·2	4	0·7–0·9 × 5–10 μ, short chains	2	∞	0·63	P
Do.	12	Good	21·1	17	0·6–0·9 × 2–4 μ, very short chains	2	∞	0·66	P
			21·2	3	0·7–1·0 × 2–5 μ, short chains	2	∞	0·95	P
Do.	12	Good	22·1	15	0·5–0·8 × 3–8 μ, granulated, long chains	2	6	0·35	P-C
			22·2	5	0·9–1·1 × 4–6 μ, very short chains	2	∞	0·55	P
Do.	8	Good	23·1	11	0·7–1·0 × 2–5 μ, singly and in pairs	3	∞	0·69	P-C
			23·2	9	0·8–1·0 × 2–5 μ, short chains	2	∞	0·90	P
Do.	14	Poor, open	24·1	13	0·7–1·0 × 2–5 μ, singly and in pairs	3	∞	0·17	P
			24·2	7	0·7–1·0 × 2–4 μ, very short chains	10	∞	2·89	B
Do.	14	Poor, bitter	25·1	4	0·8–1·1 × 4–8 μ, short chains	2	∞	0·50	P
			25·2	16	0·8–1·0 × 3–6 μ, short chains	2	10	0·66	P-C
Do.	14	Poor	26·1	8	Cocci, 0·7–1·0 μ, singly and in pairs	3	∞	2·42	Bc
			26·2	12	0·7–1·0 × 4–8 μ, short chains	2	∞	0·50	P
Do.	14	Excellent	27·1	17	0·5–0·8 × 4–8 μ, granulated, long chains	2	5	0·20	C
			27·2	3	0·8–1·1 × 3–6 μ, very short chains	4	∞	0·69	P

Manawatu	2	Poor, open	28·1	10	0·8–1·0 × 4–6 μ, short chains	2	∞	0·68	P
			28·2	4	Similar to 28·1	3	∞	0·70	P
			28·3	6	0·8–1·0 × 3–5 μ, short chains	2	∞	0·55	P
Wanganui (raw milk)	1	Very poor, open	29·1	4†	Cocci, 0·4–0·8 μ, short chains	2	11	2·33	Bc
			29·2	8	Similar to 29·1	3	15	2·69	Bc
Southland	24	Excellent	30·1	18	0·5–0·8 × 3–8 μ, granulated, long chains	2	5	0·15	C
			30·2	2	0·7–0·9 × 2–5 μ, short chains	2	∞	0·66	P
Taranaki	24	Average	31·1	16	0·6–0·8 × 2–4 μ, short chains	2	∞	0·69	P
			31·2	4	0·5–0·7 × 3–7 μ, granulated, long chains	2	8	0·25	C
Do.	12	Average	32·1	11	0·8–1·0 × 4–8 μ, very short chains	2	∞	0·76	P
			32·2	9	Similar to 32·1	2	∞	0·52	P
Taranaki (raw milk)	8	Rather poor, open	33·1	14	Cocci, 0·4–0·7 μ, short chains	2	∞	0·36	—
			33·2	6	Cocci, 0·3–0·6 μ, very short chains	3	18	2·72	Bc
Taranaki	12	Very poor, bitter, "off" flavour	34·1	4	0·7–0·9 × 3–5 μ, very short chains	2	∞	0·65	P
			34·2	6	0·5–0·8 × 2–4 μ, very short chains	5	50	0·40	P
			34·3	7	0·5–0·7 × 3–8 μ, granulated, long chains	2	4	0·26	C
			34·4	3	Cocci, 0·4–0·7 μ, pairs	2	11	0·55	—
Waikato	3	Average	35·1	5	0·8–1·2 × 3–5 μ, very short chains	3	∞	0·72	P
			35·2	11	Similar to 35·1 but chains longer	2	10	0·65	P-C
			35·3	4	0·7–1·0 × 2–4 μ, very short chains	3	∞	0·64	P
(English Cheddar) Aylburton Dairy	24	Good	36·1	16	Cocci, 0·5–0·8 μ, chains	2	∞	0·18	—
			36·2	4	0·6–1·0 × 5–8 μ, chains	2	8	0·68	P-C

* No. of strains out of the 20 isolated from each cheese which appeared to be identical on the criteria of Table I.

† The remaining 8 strains isolated from this young cheese were starter streptococci.

It is evident that *Sbm. plantarum* was by far the most common organism found in the cheeses. The genus *Streptobacterium* accounted for approximately 90% of the aggregate flora; betabacteria were more common than betacocci, while only very small numbers of other lactic acid bacteria occurred.

From the three raw-milk cheeses were isolated four strains of lactobacilli intermediate between *Sbm. plantarum* and *Sbm. casei*, eighteen strains of betacocci and thirty-eight strains which were classified in group 6. The last-mentioned strains will be discussed in later sections.

While no special endeavour was made to enumerate bacteria of types other than the lactic acid bacteria, the plates examined in every instance were either free or almost free from such organisms.

The age of the cheese did not appear to be a major factor in governing the types of lactic acid bacteria present, except during the early stages of ripening when the lactic streptococci were usually replaced by lactobacilli, and in the very late ripening stages when betabacteria frequently attained large numbers. Most of the cheeses examined in the course of this investigation fell between these two age limits; in many of the cheeses the *relative numbers* of organisms belonging to certain groups were found to alter somewhat as ripening progressed, but these quantitative changes in the flora did not appear to bear any general relation to the ages of the cheeses. As a consequence, it was considered that little would be gained in the present communication by giving the results of more than one detailed examination of each cheese.

Flora of individual cheeses. Table II shows that in good quality cheese only two to three different varieties of lactic acid bacteria (usually lactobacilli) occurred in large numbers. Poor quality cheeses often contained a larger number of different types. These points will be discussed in a later section.

More detailed examination of the organisms isolated

From the 720 strains isolated, approximately 200 strains (which included several strains from each cheese representative of the different groups present) were selected for detailed study. Among the various characteristics examined, those which were considered to be of a type most suitable for routine determination were given particular attention. In order, however, to relate the results of this investigation to classical taxonomical work it was necessary to study certain characteristics, such as the type of lactic acid formed, the determination of which involved a fairly laborious procedure. The effects of such factors as temperature, salt and *p*H were also examined on account of their importance in cheese-ripening. The data obtained permitted a detailed classification of the 200 strains to be made. The remaining 520 strains were then aligned with these by determination of those characteristics which had been found to possess the greatest differential value.

In the tables which follow, for reasons of space, only one strain representative of each variety isolated will be considered.

All the organisms studied were Gram-positive and formed small colonies on solid media. They did not form spores, produce catalase, reduce nitrate or liquefy gelatine.

Experimental

(1) *Temperature and time of incubation.* Unless otherwise indicated, all cultures were incubated at 30° C. for 14 days.

(2) *Estimation of volatile acidity.* The method employed has been described in an earlier communication (10). Probably more accurate results would have been given by the method of Hiscox *et al.* (13) or by that of Allen & Harrison (14). The method employed here, however, was considered much more suitable for routine purposes where it is necessary to examine large numbers of cultures. Check experiments in which milk containing known amounts of acetic acid and lactic acid were distilled, gave quite good recoveries of volatile acid—probably the small quantity of lactic acid which passed over tended to compensate for the amount of acetic acid retained by the protein.

(3) *Determination of the type of lactic acid produced.* The method employed was similar to that described by Hammer (15) with the exception that the concentrated whey was freed from crystalline lactose by filtration and extracted in a liquid extractor. The results obtained from determination of the percentage of water of crystallization of the zinc lactate were checked polarimetrically.

(4) *Estimation of lactose.* Farmer's modification (16) of Lane & Eynon's volumetric procedure was employed.

Morphology at different temperatures, etc. Morphology in milk at several different temperatures and on yeast-whey agar at two different temperatures was studied. Apart from the tendency for the rapid acid-producing types to stain poorly when grown in milk, and for the betacocci to assume rod-like forms on yeast-whey agar, no differentiating features were observed.

Colony form on yeast-whey agar at different temperatures. The apppearances of colonies on yeast-whey agar at different temperatures were noted. There was a definite tendency for colonies to assume "rough" forms at temperatures of 37° C. and higher, but no significant differences in colony appearance suitable for classification purposes were observed. Many strains of *Sbm. plantarum* failed to grow at 37° C. on the surface or in the depth of yeast-whey agar. This characteristic has been included in Table IV since it occasionally proved useful for distinguishing different strains and groups.

Rate of acid production in broth adjusted to different pH levels. Casein-digest broth containing 2% of lactose and 5% of yeast extract was adjusted to different *p*H values between 6·8 and 4·8. While different strains frequently showed differences in rate of growth at the various acidities, no correlation with other characteristics was observed.

Final pH and final acidity. Davis (9,17) tabulates the average final *p*H and final acidity produced by various groups of lactic acid bacteria. In the present experiments both final *p*H and final acidity in milk and in yeast-milk were

measured. An incubation period of 40 days was found to be fairly satisfactory for the organisms examined; with longer intervals the *p*H of clotted cultures tended to rise appreciably and the acidity fell to an even greater extent. The results obtained, however, did not aid particularly in the separation of the different organisms which were examined and, consequently, they are omitted from the tables.

Rate of proteolysis in milk. The rates of formation of non-protein nitrogen in milk by the various strains of lactobacilli were measured. The data obtained, however, were of little value for purposes of classification since the only correlation observed was a general one linking the rate of acid production with the rate of proteolysis (18,19).

Thermal death-point. Thermal death-points of the different strains of lactobacilli were determined in yeast-milk; certain variations were observed, but these did not appear to be significant.

Remarks on the different groups

It will be seen from Tables III–V that a more thorough examination of the characteristics of the various strains confirmed the broad classification assigned in Table II.

In the following paragraphs the different groups will be considered seriatim.

(1) *Sbm. plantarum* (20) *or Lactobacillus plantarum* (21). The 410 strains isolated were grouped in four varieties, largely on the basis of their sugar reactions.

Variety A, represented by strain no. 10·2, comprised sixty-three strains. Only a small number of sugars—six to nine out of the twenty tested—were fermented, and those only weakly. Glycerin, pentoses, rhamnose, mannite and salicin were not attacked. In this variety were included the comparatively small number of strains (27) which deviated at all seriously from typical *Sbm. plantarum* in their rate of formation of carbon dioxide.

Variety B, represented by strain no. 10·5, comprised 112 strains. Acid was formed from fifteen to sixteen sugars out of the twenty tested. Rhamnose, sorbite, saccharose and melezitose were always attacked. All the strains were highly salt-tolerant. No growth occurred on yeast-whey agar at 37° C. The amount of volatile acid formed in yeast-milk was depressed slightly by the addition of citric acid.

Variety C, represented by strain no. 3·1, comprised 121 strains. Eleven to thirteen sugars were fermented, but rhamnose, sorbite, saccharose and melezitose were never attacked. No growth occurred on yeast-whey agar at 37° C. The presence of citric acid caused a small increase in the amount of volatile acid formed. A very characteristic feature of the organisms in this variety was the pleasant aroma which they produced in yeast-milk culture, an odour reminiscent of the lower aliphatic esters being developed.

All strains of *Sbm. plantarum* which did not fall into one of the three preceding varieties (114 strains) were gathered together in variety D, repre-

Table III. *Sugar reactions of the lactic acid bacteria isolated from Cheddar cheese*

Table III. *Sugar reactions of the lactic acid bacteria isolated from Cheddar cheese*

The medium employed was casein-digest broth containing 0·5 % N. Acid formed is expressed as parts/1000 lactic acid. In the text which follows, figures above 0·9 part/1000 lactic acid are considered to indicate definite fermentation.

Strain no.	Strains with similar characteristics	Glycerin	Arabinose	Xylose	Rhamnose	Mannose	Mannite	Dextrose	Levulose	Galactose	Dulcite	Sorbite	Saccharose	Lactose	Maltose	Raffinose	Melezitose	Salicin	Dextrin	Inulin	Starch
Sbm. plantarum:																					
10·2	8·2, 10·3, 16·1, 16·2, 24·1, 28·1, 28·2, 34·2	0·0	0·4	0·7	0·4	3·8	0·4	3·8	2·9	3·4	0·4	1·6*	0·4*	2·9	3·8*	0·4*	0·4*	0·5	0·0	0·0	0·0
10·5	4·2, 10·4, 11·1, 12·1, 13·1, 13·2, 14·1, 16·3, 20·1, 20·2, 25·1, 26·2	1·3	6·5*	0·7	2·5	9·5	3·1*	8·8	8·5	7·2	0·0	4·5	7·2	8·3	8·3	7·7	3·4	7·6	1·6	0·0	0·7
3·1	1·1, 2·1, 4·3, 6·2, 7·1, 7·3, 9·1, 15·2, 17·1, 19·2, 22·2, 27·2, 28·3, 35·1	1·3	2·5*	1·4	0·5	7·7	5·2	8·1	7·6	7·0	0·5	0·3	0·5	7·4	7·6	2·9*	0·5	6·1	1·6	0·9	0·0
2·3	9·3, 11·2, 12·2, 17·2, 21·1, 21·2, 23·2, 30·2, 31·1, 34·1, 35·3, 32·1, 32·2	0·9	0·7*	0·7	0·7	3·8	3·6*	5·3	4·1	3·6	0·4	2·5*	2·8	3·7	4·8	2·9	0·9*	3·1	2·0	0·0	0·5
Sbm. casei:																					
19·1	4·1, 8·1, 30·1	0·7	0·0	0·0	0·0	2·9	0·6	2·9	2·2	2·2	0·2	0·4	0·6	4·0	0·0	0·0	0·9	0·0	0·7	0·7	0·0
31·2	5·1, 17·3, 27·1, 34·3	0·0	0·5	0·4	0·4	7·2	4·3	8·6	7·6	7·2	0·7	2·2	3·4	5·8	2·7	0·7*	0·2*	3·0	0·7	0·5*	0·5
Intermediate (*plantarum-casei*):																					
2·2	9·2, 25·2, 35·2	0·7	0·9	0·9	0·9	7·6	4·3	8·1	7·2	5·9	0·5	4·9	6·5	6·9	7·8	7·2	0·4	5·2	2·0	0·7	0·7
5·2	3·2, 18·2, 22·1, 23·1, 36·2	0·7	0·5*	0·0	0·0	4·1	2·9	7·7	7·0	6·1	0·4	0·4	4·9*	3·1	5·6	0·5*	4·1*	3·4	1·0	0·0	0·5
Betabacteria:																					
1·2	6·1, 10·1, 13·3E, 14·2, 15·1, 17·4E, 24·2	0·0	3·4	3·1	0·0	0·4	1·1	1·8	5·6	1·8	0·0	0·0	0·0*	1·8	1·6	0·4	0·0	0·4	0·4	0·0	0·0
Betacocci:																					
18·1	26·1	0·4	2·2	0·9	0·7	2·0	0·7	1·6	2·7	2·0	0·5	0·7	1·4	0·9	1·6	2·2	0·0	0·4	0·7	0·7	0·7
29·1		0·4	0·2	0·3	0·2	1·1	0·2	2·2	1·4	1·4	0·4	0·0	4·1	2·7	3·8	2·2	0·0	0·0	0·2	0·0	0·0
29·2	33·1	0·9	0·2	0·2	0·2	1·4	0·2	1·6	0·9	1·1	0·0	0·0	3·8	3·6	3·8	1·1	0·0	0·0	0·0	0·0	0·0
Other L.A.B.:																					
7·2		1·3	1·4	0·7	2·3	8·8	4·1	8·6	7·9	3·4	0·4	5·0	7·4	7·9	8·3	7·7	4·7	5·4	1·6	0·9	1·1
33·1	36·1	0·0	0·0	0·4	0·0	2·9	0·0	4·7	4·5	1·1	0·0	0·0	4·3	5·6	0·1	0·5	0·0	0·5	0·5	0·0	0·2
34·4		2·0	2·7	1·0	0·5	4·5	2·3	4·5	4·3	2·5	0·4	1·8	0·4	3·2	4·1	0·7	0·2	3·8	2·3	0·5	0·7

* Signifies fermentation by some strains but not by others.

Table IV. *Main products formed from lactose by the different varieties and growth on yeast-whey agar at* 37° C.

Strain no.	Type of lactic acid (from lactose)	Non-volatile acid formed		Volatile acid formed			Carbon dioxide formed		Growth on yeast-whey agar at 37° C.
		g./l. lactic acid	% lactose fermented	g./l. acetic acid	% lactose fermented	+0·4% citric acid (g./l. lactic acid)	g./l.	% lactose fermented	
Sbm. plantarum:									
10·2	i	7–9·5	65–80	0·2 –0·7	2·0–7·5	0·2 –1·5	0·2 –0·7	2–6	Variable
10·5	i	7–9·5	70–80	0·6 –0·8	6·0–7·5	0·4 –0·6	0·5 –0·8	5–9	0
3·1	i	7–9·5	75–85	0·6 –0·8	6·0–7·5	0·7 –0·8	0·5 –0·8	5–9	0
2·1	i	7–9·5	75–85	0·6 –0·8	6·0–7·5	0·5 –1·5	0·5 –0·8	5–9	Variable
Sbm. casei:									
19·1	d	11–14	85–95	0·15–0·30	1·0–2·0	0·15–0·30	0·15–0·25	1–2	Variable
31·2									
Intermediate (*plantarum-casei*):									
2·2	i	7–13	80–90	0·2 –1·0	2–10	0·2 –2·0	0·5 –0·8	4–9	Variable
5·2									
Betabacteria:									
1·2	i	3–6·5	35–50	0·2 –0·3	2–3	0·3 –0·4	1·0 –3·5	13–28	Good
Betacocci:									
18·1	l	4–5	35–45	0·2 –0·3	2–3	0·3 –0·4	2·5 –3·0	20–30	Good
29·1	l	4–5	35–45	0·2 –0·3	2–3	0·2 –0·3	2·5 –3·0	20–30	Good
29·2	l	4–5	40–50	0·7 –0·8	6–8·5	1·5 –2·0	2·5 –3·0	20–30	Good
Other L.A.B.:									
7·2	d	5–8	80–95	0·0	—	0·0	0·2 –0·8	3–8	Good
33·1									
34·4									

Table V. *Stimulation of representative strains by yeast extract and influence of temperature and salt on rate of acid production*

Strain no.	Milk, ° C.						Yeast-milk, ° C.						Percentage of salt							
	10	15	22	30	37	45	10	15	22	30	37	45	0	1	2	3	4	5	6	7
Sbm. plantarum:																				
10·2	0·3	0·4	1·4	1·9	1·9	0·3	1·0	3·3	5·5	7·7	7·8	0·2	7·7	7·4	7·0	7·0	6·7	6·1	5·4	3·6
10·5	0·5	1·0	2·3	5·4	4·8	0·0	1·2	5·7	9·5	9·9	8·6	0·1	9·9	10·2	9·4	9·2	8·7	8·7	8·2	8·0
3·1	0·3	1·4	2·2	3·7	3·6	0·1	0·5	5·5	8·4	9·8	7·1	0·2	9·8	10·6	10·1	10·0	9·6	7·6	6·1	5·3
2·3	0·3	1·2	1·7	4·2	2·3	0·0	0·3	1·6	4·7	10·7	7·7	0·2	10·7	10·8	10·0	9·0	8·1	4·9	4·0	2·8
Sbm. casei:																				
19·1	0·9	2·9	7·8	10·3	5·5	0·1	2·3	7·5	11·4	12·4	10·9	0·3	12·4	11·2	9·8	8·9	6·4	4·1	1·5	0·0
31·2	1·4	4·6	10·0	13·3	11·3	1·4	2·6	8·3	12·0	14·0	12·5	3·2	14·0	12·9	10·4	8·3	7·5	6·5	4·0	1·9
Intermediate (*plantarum-casei*):																				
2·2	0·4	1·4	4·8	8·3	4·1	0·0	1·1	5·4	7·2	9·9	8·6	0·1	9·9	10·7	10·4	9·7	8·9	8·8	7·9	7·7
5·2	0·8	1·9	6·8	7·7	4·8	0·5	1·0	4·2	7·7	8·5	8·2	0·5	8·5	8·3	7·6	7·0	4·9	3·5	3·3	0·0
Betabacteria:																				
1·2	0·1	0·3	1·0	1·9	2·5	0·0	0·4	1·7	5·6	7·1	6·0	0·1	7·1	7·2	7·0	4·6	3·2	1·4	0·5	0·0
Betacocci:																				
18·1	0·1	0·5	0·7	1·7	0·9	0·2	0·2	3·2	4·1	5·9	4·6	0·2	5·9	6·8	6·2	5·4	5·3	4·9	3·9	3·7
29·1	1·5	2·6	3·3	4·6	3·6	0·0	2·3	5·2	6·7	5·4	3·7	0·1	5·4	5·0	4·6	3·8	2·2	2·0	0·0	0·0
29·2	0·9	1·3	2·0	3·2	2·8	0·0	1·7	4·3	6·1	5·0	3·2	0·2	5·0	4·1	2·7	2·3	0·9	0·8	0·0	0·0
Other L.A.B.:																				
7·2	0·0	0·9	3·0	6·0	7·2	5·2	0·0	3·3	5·7	6·8	7·2	7·4	6·8	6·7	5·9	5·4	3·4	0·1	0·1	0·0
33·1	0·0	0·6	3·4	7·0	7·5	5·3	0·0	2·2	5·5	7·5	7·7	6·8	7·5	6·8	6·5	5·4	0·1	0·1	0·0	0·0
34·4	0·7	3·4	4·0	4·8	4·0	2·5	1·4	4·8	5·1	5·2	4·6	3·4	5·2	4·2	3·8	3·3	2·6	2·0	1·9	1·4

sented by strain no. 2·3. This variety, however, formed a homogeneous group which, as will be seen from its sugar reactions (Table III), appeared to occupy a position intermediate between the two preceding varieties—some strains, e.g. nos. 32·1 and 32·2, were quite closely related to variety B, since they attacked rhamnose, saccharose and melezitose.

(2) *Sbm. casei*. The 106 strains were placed in two varieties (A and B) which showed differences in salt tolerance and in sugar reactions.

Variety A, represented by strain no. 19·1, comprised fifty-five strains; it was very sensitive to salt and it fermented only a small number of sugars (five to six, and those only weakly).

Variety B, represented by strain no. 31·2, comprised fifty-one strains. This variety was much more salt-tolerant than variety A and it fermented ten to thirteen sugars.

In general, *Sbm. casei* converted a larger proportion of lactose to non-volatile acid than did *Sbm. plantarum*, but it produced carbon dioxide and volatile acid at about only one-third the rate. *Sbm. casei* was not stimulated by yeast extract at the optimum growth temperature, but definite stimulation was noted at lower temperatures. At comparatively low temperatures *Sbm. casei* was restrained less than any of the other organisms examined.

(3) *Strains intermediate between* Sbm. casei *and* Sbm. plantarum. These seventy-eight strains were grouped in two varieties. Variety A, represented by strain no. 2·2, comprised thirty-six strains; it fermented only five to six sugars out of the twenty tested and was highly salt-tolerant.

Variety B, represented by strain no. 5·2, comprised forty-two strains; it fermented ten to thirteen sugars and was much less tolerant of salt than variety A.

The group resembled *Sbm. casei* in some respects, e.g. rate of acid production and reduction of litmus milk, but the chief resemblance was to *Sbm. plantarum*.

(4) *Bbm. breve or Lactabacillus brevis* (22). The thirty-nine strains of betabacteria were grouped in one variety of *Bbm. breve*. Sugar reactions were typical. An interesting point was the very decided preference shown for levulose as contrasted with dextrose. All strains grew on yeast-whey agar at 37° C., were stimulated more than *Sbm. plantarum* by yeast extract, and were inhibited by low temperatures and high salt concentrations. Volatile acid was formed somewhat more slowly than in the case of *Sbm. plantarum*. The rate of gas production was approximately three times that of *Sbm. plantarum* and nine times that of *Sbm. casei*.

(5) *Betacocci* (*Leuconostoc*). These thirty-eight strains were classified most satisfactorily on the scheme of Hucker & Pederson (23). The twenty strains represented by strain no. 18·1 were grouped in one variety of *Leuconostoc mesenteroides*; it produced a relatively low final *p*H in milk, fermented arabinose, levulose, dextrose, mannose, galactose, saccharose, raffinose and lactose and displayed a comparatively high salt tolerance.

Slightly more common were the two varieties (A and B) of *Leuconostoc dextranicus*, represented by strains nos. 29·1 (four strains) and 29·2 (twenty-two strains) respectively. These two varieties fermented sucrose, dextrose, galactose, maltose, levulose, lactose, mannose and raffinose; they produced less acid than did the strains of *Leuconostoc mesenteroides* and were more sensitive to salt. The main difference between varieties A and B lay in the amounts of volatile acid formed. Certain other differences between these two varieties will be discussed in a later section.

It is remarkable that *Leuconostoc citrovorus*, common in starter cultures, was not isolated from any of the cheeses.

Organisms of group 6. The twenty-nine strains of the remaining group all formed *d*-lactic acid and produced only small amounts of by-products.

The four strains, represented by strain no. 7·2, fermented pentoses, maltose, dextrin, salicin, sorbite and glycerin; they were classified as *Str. glycerinaceous* (20).

Thirty strains were represented by strain no. 33·1; they fermented saccharose, but did not attack maltose, dextrin, salicin and pentoses, their maximum growth temperature was $>45°$ C. and salt tolerance was low; these strains were classified as *Str. thermophilus*.

The three strains, represented by strain no. 34·4, occurred in the form of diplococci, fermented maltose, dextrin, salicin and pentoses, but did not attack saccharose, raffinose or rhamnose; their maximum growth temperature was $<45°$ C.; these organisms were grouped as *Str. lactis*.

The influence of different strains of lactobacilli and betacocci upon cheese quality. Selected strains from the different types studied were added to the milk employed in cheese-making. The methods of cheese manufacture, etc. used have been described in earlier communications (10, 12). In these publications, too, the effect of a number of different strains of lactic acid bacteria upon the flavour and upon the texture of cheese have been separately considered. A given strain of lactobacilli may, however, influence cheese quality in several different ways simultaneously. Flavour, body and colour (where annatto is used) may all be affected. In Table VI the influence of the various strains upon the flavour and the colour of cheese is shown. The influence upon cheese body has not been included since the effects of most strains appear to be much the same, the body breaking down more rapidly in the inoculated cheeses than in the controls. A similar result with *L. casei* has been reported recently by Lane & Hammer (24).

It is evident that the addition to the cheese-milk of *Sbm. casei*, organisms intermediate between *Sbm. casei* and *Sbm. plantarum*, and one variety of *Sbm. plantarum* improved the quality of the cheese. On the other hand, defects were caused by betabacteria, betacocci and two varieties of *Sbm. plantarum*. (One variety of *Sbm. plantarum* had little apparent influence on cheese quality.) The defects produced included bad flavours, open texture and discoloration.

Discoloration in New Zealand Cheddar cheese

The type of discoloration which occasionally affects New Zealand cheese coloured with annatto occurs round slits or other openings in the cheese. Several forms of the defect—"bleached", "pink" and "muddy"—have been observed. Morgan(25) showed that the presence of air was necessary for the appearance of discoloration and he attributed the defect to the action of moulds. The chemistry of discoloration was investigated by two workers: Moir(26) considered that the chemical processes involved in the development of bleached, pink and muddy discoloration were, respectively, reduction, action of acids and action of mould enzymes; Barnicoat(27), as a result of his experiments, concluded that the mechanism underlying the development of both bleached and pink discoloration was an oxidation process catalysed by sulphydryl.

Apart from any consideration of the mechanism of the discoloration of annatto in cheese, it was evident in the course of the present investigation that inoculation of cheese milk with certain strains of lactobacilli led regularly to the occurrence of discoloration in the cheese.

It will be seen from Table VI that discoloration was caused by all strains of *Bbm. breve* studied, most strains of betacocci and two varieties of *Sbm. plantarum*. It is apparent, moreover, that a given single strain belonging to these types may produce more than one form of discoloration, e.g. strain no. 13·3 E produced both bleached and muddy discoloration. This probably explains why, during a serious outbreak of discoloration which occurred in 1930–1, all three forms of the defect were prevalent.

Discussion

The aggregate flora of Cheddar cheese

In the preceding sections it has been shown that the flora of New Zealand Cheddar cheese consists almost entirely of lactic acid bacteria. This accords with the observations of Davis(9) on the flora of Cheddar cheese made from clean milk. After the relatively short interval immediately following manufacture during which the starter streptococci predominate, lactobacilli, and less commonly, betacocci, flourish over the major portion of the ripening period. Of the lactic acid bacteria isolated from New Zealand pasteurized-milk cheese during the present investigation, 62% were *Sbm. plantarum*, 16% were *Sbm. casei*, and strains intermediate between these two species accounted for 12%; thus 90% of the flora consisted of streptobacteria. Betabacteria (6%) and betacocci (3%) occurred in smaller numbers, leaving only 1% for other types of lactic acid bacteria (*Str. lactis*, *Str. thermophilus*, etc.).

The dominance of *Sbm. plantarum* in New Zealand Cheddar cheese is somewhat surprising in view of the important role in cheese-ripening assigned by earlier workers to *Sbm. casei* and to different types of cocci. From the recent

Table VI. *The effect of different strains of lactobacilli and betacocci upon cheese quality*

Strain no.	No. of cheeses manufactured	Apparent effect produced by added organisms* (0 signifies no apparent effect) Flavour	Texture	Colour
Sbm. plantarum:				
Variety A				
8·2	2	Somewhat unclean, "sulphide"	0	Bleached, pink
10·2	2	Unclean, bitter	0	Bleached
16·1	2	Unclean, "sulphide"	0	Bleached
16·2	2	Unclean, "sulphide"	0	?
24·1	2	Unclean, "sulphide"	0	?
28·1	2	Unclean, "sulphide"	0	Bleached
Variety B				
11·1	2	Bitter	0	Bleached, pink
12·1	2	Bitter	0	Bleached, pink
13·2	2	Unclean	0	?
16·3	2	Unclean, "sulphide"	Slightly open	Bleached, pink
20·1	27	Bitter, unclean	Open	Bleached, pink
Variety C				
1·1	34	Improved	0	0
3·1	2	Improved	0	0
4·3	2	Improved	0	0
6·2	4	Improved	0	0
7·3	2	Improved	0	0
Variety D				
21·1	4	Slightly improved	0	0
21·2	2	0	0	0
23·1	2	0	0	0
31·1	2	0	0	0
32·1	2	Slightly unclean	0	0
32·2	2	Slightly unclean	0	0
Sbm. casei:				
Variety A				
8·1	4	Slightly improved	0	0
19·1	2	Slightly improved	0	0
Variety B				
5·1	6	Slightly improved	0	0
27·1	2	Slightly improved	0	0
31·2	2	Slightly improved	0	0
Intermediate (*plantarum-casei*):				
Variety A				
25·2	4	0	Slightly open	0
Variety B				
3·2	4	Slightly improved	0	0
5·2	2	Slightly improved	0	0
22·1	4	Slightly improved	0	0
23·2	3	Slightly improved	0	0
Betabacteria:				
1·2	6	"Fermentation", yeasty	Open	Bleached, pink
10·1	2	Unclean, "sulphide"	Open	Bleached
13·3 E	20	"Fermentation", yeasty	Very open	Bleached, muddy
14·2	2	"Fermentation", yeasty	Open	Bleached
15·1	2	Unclean, "earthy"	Open	Bleached
17·4 E	2	"Fermentation", yeasty	Slightly open	?
Betacocci (*Leuconostoc*) *Leuconostoc mesenteroides*:				
33·2	3	Tart, bitter	Open	Bleached, pink
Leuconostoc dextranicus:				
Variety A				
29·1	3	Slightly improved	Open	0
Variety B				
29·2	7	Bitter	Open	Bleached

* Control cheeses, made each day from a portion of the milk, taken as standard reference.

data of Davis[9], on the frequency distribution of various types of lactic acid bacteria in English Cheddar cheese made from raw milk of low bacterial content, it would appear that *Sbm. plantarum* is usually present in a weakened condition and does not occur after the cheese is more than five months old, the flora then consisting entirely of *Sbm. casei*. In New Zealand cheese, on the other hand, weakened forms of *Sbm. plantarum* do not seem to occur in any appreciable numbers. Normally, vigorous varieties of *Sbm. plantarum* dominate the flora over the major part of the ripening period tending to be displaced only in the final stages of maturity, and then not by *Sbm. casei* but by betabacteria. This change in the flora during the advanced stage of maturity suggests that possibly the characteristic strong flavour ("bite") associated with old cheese is correlated with the development of the betabacteria (which are known to produce strong flavours).

The routine identification of lactic acid bacteria isolated from cheese

The disadvantages inherent in Orla Jensen's procedure for the identification of lactic acid bacteria have been discussed by Davis[17] who outlines a more suitable routine method. In the present investigation a smaller range of types has been encountered and a very simple method involving the determination of only four characteristics—morphology and the rates of coagulation of yeast-milk, reduction of litmus milk and evolution of carbon dioxide—has been shown to be very satisfactory for the identification of *Sbm. casei*, *Sbm. plantarum*, organisms intermediate between these two species, betabacteria and betacocci.

The flora of individual cheeses, and the correlation of certain readily determined biochemical characteristics of different organisms with the effects which these organisms produce in cheese

It has already been shown (Table II) that whereas only two or three different varieties of lactic acid bacteria are isolated from cheeses of good quality, poor quality cheeses often contain a more diverse flora.

From preceding tables it will be seen that the effects which different types of lactobacilli and betacocci produce in cheese are clearly related to their biochemical characteristics. In the following discussion it will be convenient to deal with each group of organisms in turn.

Sbm. plantarum. Different strains of *Sbm. plantarum* were found to produce widely different effects in cheese. It will be observed, however, that the effects of the various strains upon cheese quality were correlated very closely with the division of these organisms into the four varieties discussed in an earlier section. All organisms of varieties A and B produced objectionable flavours and discoloration in cheese. The most salt-tolerant strains also caused open texture to develop. Variety C invariably produced desirable cheese flavours. Variety D, in keeping with its affinities to both varieties B and C, appeared to produce an "average" type of cheese. It will be noted that strains of variety D (e.g.

strains nos. 32·1 and 32·2) which showed a marked resemblance to variety B were capable of exerting a slightly undesirable influence upon cheese quality.

Sbm. casei. Unfortunately, only a comparatively small number of strains of *Sbm. casei* were employed in cheese-making experiments. While certain strains were found to produce a smaller effect than others, none proved actually detrimental to the quality of the cheese.

Intermediate organisms (plantarum-casei). Organisms intermediate between *Sbm. casei* and *Sbm. plantarum* did not appear to produce any undesirable flavours in cheese (Table VI), but the more salt-tolerant variety (A) caused the appearance of open texture.

Betabacteria. Earlier investigations(10,11,12), and the results of cheese-making experiments described in this paper (Table VI), have shown that the presence of large numbers of betabacteria in cheese are detrimental to its quality. Open texture is almost invariably produced and frequently discoloration and objectionable flavours develop. On the other hand, there is good reason for believing that *a small proportion* of betabacteria in the flora actually improves cheese flavour. A number of the best cheeses examined have been found at an age of 2–3 months to contain an appreciable percentage of betabacteria. As mentioned in an earlier publication(10), it is known that practically all mature cheeses contain a certain number of these organisms.

The marked retarding influence of salt and low temperatures on the rate of growth of betabacteria is of immediate practical importance. Low temperatures of curing and relatively high salt content are both known to be inimical to the development of open texture and strong flavours in cheese(28,29), and the effect has often been attributed to a general reduction in the rate of bacterial growth under these conditions. From Table V it will be seen, however, that the betabacteria are inhibited to a greater degree than the other groups of lactic acid bacteria examined. Consequently, it would appear that the closer texture and milder flavour of cheese carrying a high percentage of salt and cured at low temperatures are conditioned more by the selective influence of these factors upon the betabacteria than by a general restraining influence on all the organisms present.

Betacocci. Evans(5) described experiments in which certain strains of betacocci added to pasteurized cheese-milk brought about an improvement in the flavour of the cheese. In similar work carried out at this Institute the results have not been so well defined. Occasionally cheeses of normal "make" have been found to contain large numbers of betacocci, but when cheese-milk has been inoculated with these organisms only poor growth in the cheese has usually resulted. The cheese manufactured during the present experiments (Table VI) contained a heavy inoculum of betacocci but, nevertheless, the results obtained must be accepted with some reservation, since the added betacocci did not flourish. In general, betacocci would seem to have an influence similar to that of the betabacteria. There was some evidence, however, that *Leuconostoc dextranicus* variety A produced a desirable flavour in cheese.

This variety differed from variety B in the relatively larger amount of non-volatile acid which it formed.

In this connexion, too, it is of interest to note that the desirable flavour-producing varieties C and D of *Sbm. plantarum* also formed a somewhat higher proportion of non-volatile acid than did the objectionable varieties A and B, indicating, possibly, that the two former were more closely related to *Sbm. casei* than were the latter.

Remarks on the classification of the genera Streptobacterium *and* Betabacterium

Orla Jensen (20) places *Sbm. plantarum* and *Sbm. casei* in the same genus but allocates the "gas-producing" lactobacilli to a separate genus. This would indicate a wider difference between the betabacteria and *Sbm. plantarum* than between *Sbm. plantarum* and *Sbm. casei*.

In the present experiments a large number of simply determined characteristics of different strains of lactobacilli isolated from cheese have been investigated. The strains examined have been classified as *Sbm. casei*, *Sbm. plantarum* and *Bbm. breve* (other species of betabacteria were not isolated). From a consideration of the following characteristics of these groups it will be seen that there is a gradation from *Sbm. casei* through *Sbm. plantarum* to *Bbm. breve*, *Sbm. plantarum* occupying an intermediate position:

(1) *Morphology.* *Sbm. plantarum* falls somewhat closer to *Bbm. breve* than to *Sbm. casei*.

(2) *Type of lactic acid.* Again, *Sbm. plantarum* resembles *Bbm. breve* more closely than *Sbm. casei*.

(3) *Formation of by-products.* This is an interesting point, since it was largely on the basis of formation of gas and other by-products that Orla Jensen separated the genus *Betabacterium* from the genus *Streptobacterium*.

(*a*) *Rate of formation of carbon dioxide.* *Bbm. breve* produces carbon dioxide approximately three times as rapidly as does *Sbm. plantarum* which, in turn, produces gas at about three times the rate of *Sbm. casei*.

(*b*) *Amount of volatile acid formed.* *Sbm. casei* forms only very small amounts of volatile acid, but *Sbm. plantarum* produces practically as much as does *Bbm. breve*.

(4) *Rate of acid production in milk.* *Sbm. casei* produces acid more rapidly than does *Sbm. plantarum* which, in turn, is a faster acid producer than *Bbm. breve*.

(5) *Stimulation by yeast extract.* *Sbm. casei* is not stimulated at the optimum growth temperature, *Sbm. plantarum* is definitely stimulated at all temperatures, while the degree of stimulation of *Bbm. breve* is even greater.

(6) *Retardation of growth at low temperatures of incubation.* *Sbm. casei* is least retarded, *Bbm. breve* most retarded.

(7) *Influence upon cheese quality.* *Sbm. casei* is almost always beneficial,

Sbm. plantarum is often beneficial, but *Bbm. breve* is usually detrimental (except when present in small numbers).

It is realized that care is necessary in drawing conclusions from the above results since only a relatively small number of varieties, all isolated from Cheddar cheese, have been studied. It would appear, however, that the American grouping of the "gas-producing" lactobacilli as species of the genus *Lactobacillus* (22), and of the "non-gas-producing" rods isolated from fermenting substances as another species of the same genus (21) is more satisfactory than Orla Jensen's system, but neither scheme indicates sufficiently the gradations which exist between the groups in question.

Remarks on the possible sources of lactobacilli. From work already described it is clear that commercial Cheddar cheese frequently contains large numbers of undesirable types of lactobacilli. The question arises, therefore, as to whether it is possible to prevent these types from gaining access to the cheese-milk. Before this question can be answered, an investigation of the sources of lactobacilli in general is necessary. Accordingly, experiments along these lines have been undertaken in this Institute; the work, however, has not yet progressed beyond the preliminary stages. One point arising from the present investigation, however, which bears on the problem may be noted.

Although it would seem that many types of lactobacilli are very widespread, there is strong evidence that certain varieties can become so firmly established in specific localities—whether in the factory, in the milking equipment or on the pastures—that they regularly gain access to the cheese-milk over long periods of time. For example, strain no. 10·5, described above, has been regularly isolated from cheese made in the Institute factory during the last five years. It is suggested that many "district" characters observed in cheese may arise in this way, the same types of lactobacilli regularly passing into the cheese produced in a given factory and causing different cheeses manufactured over long periods to develop the same character.

SUMMARY

Of the lactic acid bacteria isolated, 720 strains from thirty-six typical Cheddar cheeses have been classified. The dominant organism in New Zealand cheese has been shown to be *Sbm. plantarum*. *Sbm. casei* occurs much less frequently, while betabacteria and betacocci are found in still smaller numbers. Betabacteria tend to replace *Sbm. plantarum* in the final stage of cheese-ripening. The flora of good quality cheese has been found to consist mainly of one or two varieties of *Sbm. plantarum*, often associated with *Sbm. casei* or a small proportion of betabacteria. In poor-quality cheese a larger number of types of lactic acid bacteria frequently occur. A simple routine method is described for distinguishing the different groups present in cheese.

The effects which different strains of lactobacilli and betacocci produce in cheese have been correlated with their biochemical characteristics. *Sbm. casei*

in general appears to be beneficial to cheese quality. The strains of *Sbm. plantarum* isolated have been grouped in four varieties, mainly on the basis of sugar reactions; one variety has been shown to be beneficial to cheese quality, another variety has very little apparent effect, while the remaining two varieties produce serious defects—bad flavours, discoloration and, occasionally, open texture. The same defects are produced by most strains of betabacteria and betacocci when present in large numbers.

The biochemical characteristics of the lactobacilli studied have shown a more regular gradation from *Sbm. casei* through *Sbm. plantarum* to *Bbm. breve* (the only species of *Betabacterium* isolated) than is indicated from the generally accepted systems of classification.

The author is indebted to Prof. W. Riddet and Dr H. R. Whitehead for their interest in this work, and for suggestions and criticism. Acknowledgement is also made of the assistance given by the staff of the Institute factory in the manufacture and examination of the inoculated cheeses.

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237. LACTIC ACID BACTERIA IN RELATION TO CHEESE FLAVOUR

II. OBSERVATIONS ON THE INOCULATION OF THE MILK EMPLOYED IN CHEESE MANUFACTURE WITH LACTOBACILLI

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Lactobacilli have recently been shown to play a very important part in the development of flavour in Cheddar cheese made from pasteurized milk(1,2,3). In the literature many investigations upon cheese flavour in which different types of lactobacilli were added to the milk employed in cheese manufacture are reported. Evans *et al.*(4) found that inoculation of pasteurized cheese-milk with several strains of lactobacilli led to the development of sour flavours in the cheeses during the early stages of ripening. Davies *et al.*(5) in a similar investigation, employing raw milk, were unable to detect any flavour changes in the cheeses due to the addition of cultures of *Sbm. casei* and of *Sbm. plantarum*. Lane & Hammer(1) inoculated cheese-milk pasteurized at 145° F./30 min. with strains of *Sbm. casei* and found that the flavours of the resulting cheeses were better than those of the control cheeses, although many of the flavours were "not typically Cheddar". Investigations(2,3) carried out in this Institute on the addition of strains of *Sbm. casei*, *Sbm. plantarum*, betabacteria and betacocci to milk pasteurized either by holding at 143–145° F./30 min. or by the flash method (temperatures ranging from 150 to 175° F.) showed that betabacteria, betacocci and two varieties of *Sbm. plantarum* produced objectionable flavours in cheese. On the other hand, it was found that *Sbm. casei* (in agreement with the work of Lane & Hammer) and one variety of *Sbm. plantarum* improved cheese flavour.

It is proposed in this paper, to devote attention to several points of interest in connexion with the actual process of inoculation of the cheese-milk with lactobacilli. In the first section the influence of variations in the size of the inoculum upon cheese flavour will be discussed and experiments will then be described which have been carried out in attempts to find methods suitable for the propagation of strains of lactobacilli in cheese factories.

Influence of size of inoculum

In investigations previously carried out by different workers on the influence of lactobacilli upon cheese quality the object was to observe the broad effects brought about by different strains. Consequently, in order to be

reasonably certain that the added types would predominate in the flora of the cheese, the cheese-milk was inoculated very heavily with the different cultures, e.g. Lane & Hammer used 1·5% (calculated on the cheese-milk) of a milk culture of *Sbm. casei*. On the assumption that, say, 500,000,000 lactobacilli were present in each ml. of culture, the lactobacillary content of the cheese-milk would be approximately 7,500,000/ml. In early experiments carried out at this Institute, inocula up to 0·2% were employed; consequently, while the amounts of different cultures added to the cheese-milk were smaller than those used by other workers, the actual numbers of organisms added were still, in most instances, very much greater than the numbers of lactobacilli which one would expect to find in ordinary milk used for cheese-making. The question arises, therefore, as to whether the extremely large inocula used would be likely to cause the ripening changes in the cheese to follow a slightly abnormal course. Some evidence pointing to an effect of this kind was provided by the results of earlier experiments(2), in which it was noticed that a peculiar "acid" flavour frequently appeared in the inoculated cheese during the early stages of maturation. Evans *et al.*(4) reported a similar finding—the flavour being described as "sour". The "acid" flavour noted in our immature cheese frequently persisted throughout ripening, so that the mature cheese often possessed flavours which were rather attractive but somewhat marred by sharpness; while the "bodies" of the cheese showed characteristics usually associated with an excess of acid.

The addition of very large numbers of streptobacteria to the cheese-milk may conceivably affect the ripening process of cheese in two ways. The normal floral sequence may be affected. For example, the streptobacteria inoculated may displace the lactic streptococci (from the starter) too early, or they may tend to "crowd out" other necessary organisms. There is some evidence for the view that betabacteria (and sometimes betacocci) play a part in the development of typical Cheddar flavour(2,3). In the late stages of ripening betabacteria almost always seem to be present and they possibly contribute a mellowness to the flavour which might otherwise be lacking.

In the present investigation the effect upon cheese flavour of varying the numbers of streptobacteria added to the cheese-milk was studied. Strains of *Sbm. casei* and of *Sbm. plantarum* variety C(3) were employed. Cheeses made from milk lightly inoculated with any of these strains were found to possess better flavours than cheeses made from milk heavily inoculated with the same strains. The best results were obtained when the inoculum was such that the numbers of lactobacilli added were not much greater than the numbers of such organisms "naturally" present in raw milk, e.g. 10 ml. of clotted culture to 80 gallons of cheese-milk. On the supposition that 1 ml. of the culture contained roughly 500,000,000 organisms, this inoculum would correspond to about 12,000 organisms/ml. cheese-milk. In experiments carried out over several seasons, cheeses made with the addition of lactobacilli in this proportion proved superior to cheeses manufactured from milk inoculated with very large

numbers of the same organisms. Not only did peculiar "acid" flavours occur far less frequently in the immature cheeses, but the mature cheeses, also, developed more desirable flavours.

A CONVENIENT METHOD FOR THE PROPAGATION OF LACTOBACILLI IN CHEESE FACTORIES

When experiments carried out in the Institute factory showed that *Sbm. casei* and at least one variety of *Sbm. plantarum* exerted a beneficial influence upon cheese flavour, consideration was given to the possibility of applying the results in commercial practice. Before this could be done, however, it was clear that some simple method would have to be devised for the propagation of lactobacilli and for their addition to the cheese-milk under factory conditions. Preferably, the procedure should be one which did not in any way disturb the daily factory routine. Several possible methods were suggested:

(1) The preparation of dry powdered cultures of lactobacilli.

(2) The incorporation of lactobacilli in the liquid rennet which is used in factories.

(3) The incorporation of lactobacilli in the "starter" cultures.

The first method was troublesome and costly and, consequently, the other alternatives were considered first. Trials in which lactobacilli were added to the rennet showed that these organisms died off too quickly for the method to be practicable. Consideration, therefore, was given to the third procedure—the incorporation of lactobacilli in starter cultures.

In planning preliminary experiments it was anticipated that *Sbm. casei* would prove more suitable than *Sbm. plantarum* since the former grew much faster in milk, especially at low temperatures(3). Trials in which these two species were added to milk cultures of pure strains of *Str. cremoris* showed, however, that *Sbm. casei* survived only a few days—no longer, in fact, than did *Sbm. plantarum*. Several modifications of the method were then tried. The most successful consisted in the addition to the milk of a growth stimulant, such as yeast extract. In this enriched milk *Sbm. plantarum* grew better in association with *Str. cremoris* than did *Sbm. casei*. In the following paragraphs consideration will be limited to the one variety of *Sbm. plantarum* represented by strain no. 3·1 described in the preceding paper(3).

Changes in the lactobacillary population of the various "composite" starters were followed, at first, by a modification of the Breed method. This, however, did not prove a very satisfactory procedure, since difficulty was often experienced in distinguishing the rods (present in relatively small numbers) from elongated cocci. Consequently, a plating method was tried. In preliminary work use was made of several different agar media, including the whey medium buffered by potassium citrate and citric acid to *p*H 4·2 described by Davis(6). On this medium the starter streptococci did not grow, but as was

found by Allen & Harrison (7), the counts of lactobacilli were very low. Much better results were given by use of the following medium which had been developed earlier during a study of the types of lactobacilli present in milk:

	%
Peptone	0·5
Yeast extract	5·0
Sucrose	1·0
Arabinose	0·5
Agar	4·0

(For the present purpose, maltose could be substituted for sucrose and arabinose.)

The medium was adjusted to *p*H 5·1 and sterilized in an autoclave (12 lb.). *Str. cremoris* was completely inhibited on this medium while the counts of *Sbm. plantarum* were very satisfactory, as will be seen from the comparison with two other media given below, in which counts were made on a two-day old yeast-milk culture of *Sbm. plantarum* (strain no. 3·1):

Medium described above	390,000,000
Dextrose-bean-agar	490,000,000
Tomato-whey-agar	430,000,000

In general, the strains of *Sbm. plantarum* employed in these experiments were found to grow quite well in association with different strains of *Str. cremoris* in milk containing yeast extract, the numbers of the former organisms present in the culture decreasing only gradually with time, e.g. an initial count of approximately 40,000,000 lactobacilli/ml. on a 20-hr. culture fell to 10,000,000–20,000,000/ml. after daily propagation for three weeks. Certain strains of *Str. cremoris*, however, were encountered which did not permit the coincident growth of *Sbm. plantarum* so well as this.

The milk was usually enriched with yeast extract, but extracts of tomato, lucerne, clover, etc., also proved satisfactory. In fact, lucerne extract appeared to give rather better results than yeast extract, as will be seen from the following counts of *Sbm. plantarum* made on a composite starter which had been propagated for three weeks in milk with 5% of these different extracts (standardized to the same N content of 1·0%).

Culture medium	Count of *Sbm. plantarum*
Milk	1,000
Milk + yeast extract	12,000,000
Milk + tomato extract	12,000,000
Milk + clover extract	8,000,000
Milk + lucerne extract	18,000,000

Trials of composite starters

It was known from work already reported (3) that the variety of *Sbm. plantarum* used in this investigation exerted a beneficial influence on cheese flavour, but the "composite" starters were tried out in cheese-making experiments in the Institute factory, partly as a check on the earlier results and partly to see whether the effects produced in cheese by the lactobacilli were modified

by the different acid-producing characteristics of various strains of *Str. cremoris*. In each trial the "bulk" starter was prepared in unenriched milk; the content of lactobacilli/ml., therefore, was less than that of the mother culture—usually about one-quarter. Thus, the actual numbers of lactobacilli added to the vat corresponded to less than 50,000/ml. cheese-milk—quite a suitable inoculum. The usual precautions(2,3) were taken to avoid contamination of the control vats with lactobacilli from the inoculated vats.

Examination of the mature cheeses showed that, in general, the cheeses made with the use of "composite" starters had better flavours than those made with the use of the corresponding pure strains of *Str. cremoris*. It was further observed that the same strain of *Sbm. plantarum* gave rather better results when used in conjunction with some of the *Str. cremoris* strains than with others. Probably, this was due to the cheese-making conditions in the former case providing more suitable substrates for the activities of the added lactobacilli, since it was known that the acid-producing characteristics of the different strains of *Str. cremoris* were dissimilar.

Two "composite" starters were used commercially on a few occasions, and they were found to produce cheese of very satisfactory quality. Unfortunately, it proved extremely difficult to carry out adequately controlled experiments under commercial conditions, owing to the ease with which contamination occurred from vat to vat. As a matter of fact, however, it is considered unlikely that "composite" starters used in commercial cheese-milk would at the present time give a significant improvement in the flavour of the cheese. There must almost always be present in the bulk of the milk delivered to cheese factories a number of different types of lactobacilli which, given the requisite conditions for growth in the cheese curd, will produce characteristic flavours; consequently, it is doubtful whether the addition to such milk of comparatively small numbers of lactobacilli on the lines discussed above would be sufficient to influence the flavour of the cheese markedly in the majority of cases.

There is, however, an undoubted gradual improvement taking place in the bacteriological quality of the milk delivered to cheese factories in New Zealand and clearly, therefore, it will be essential in the future to make additions of selected organisms if the full Cheddar flavour is to be developed in the cheese. The work described above is, consequently, necessary as a preparation for the time, perhaps not far distant, when the development of cheese flavour can no longer be left to chance but must be consciously controlled—just as in the past the necessity arose for the use of starters in order that acid development in the vat could be properly controlled.

SUMMARY

Attempts to improve the flavour of cheese by inoculation of good quality cheese-milk (pasteurized) with selected strains of streptobacteria gave the best results when relatively small inocula were employed. Large inocula imparted

a sharpness to the flavour of the cheese during the early stages of ripening, and this sharpness often persisted when the cheese was mature.

Several possible methods suitable for the propagation of lactobacilli under factory conditions were investigated. The most convenient procedure consisted in the incorporation in the starter "mother" culture (enriched with a vegetable extract) of suitable strains of *Sbm. plantarum*; these strains were found to grow better in association with some starters than with others.

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238. THE EFFECT OF *p*H ON GROWTH AND GAS PRODUCTION BY STREPTOCOCCI AND LACTOBACILLI

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Although hydrogen-ion concentration is one of the most important factors governing the growth of micro-organisms, no systematic study of the effect of *p*H on the lactic acid bacteria (streptococci and lactobacilli) appears to have been made. Such information is desirable, not only because of the economic importance of the subject in relation to milk products and fermented food stuffs generally (1,2) but also from the aspects of diagnostic culture (3,4) and selective media (5,6). Gas production and the ability to form other by-products are not the least important properties of lactic acid bacteria from the economic point of view, and it is of interest to note that what may be highly desirable in one foodstuff or fermented product may be the cause of serious faults in another. Thus, for example, carbon dioxide production in fermented milk drinks is a much desired asset; in hard cheese it may be the cause of cracks and gas holes (7).

Experimental technique

An enriched casein digest medium was used in which most lactic acid bacteria grow well, thus allowing comparison of all types on the one medium. In the first experiment adjustment of *p*H was made before sterilization with the result that great destruction of the sugar took place in the alkaline range, causing a darkening of the medium and lowering the *p*H. Moreover, during incubation, continuous absorption of atmospheric carbon dioxide led to a further fall in *p*H of the alkaline media. In the second experiment these difficulties were largely eliminated by sterilizing all the media at *p*H 6·8 and adding the sugar and the calculated quantity of acid or alkali to adjust the *p*H and tubing aseptically. Carbon dioxide absorption during incubation was greatly reduced by packing the tubes in sealed tins containing soda lime in open Petri dishes.

The double digest casein broth used (8) contained *c.* 0·35% total nitrogen plus 5% yeast autolysate (9), 2% dextrose and 0·25% phosphate. (Equivalent molar amounts of KH_2PO_4 and Na_2HPO_4.)

Exp. I. A buffer curve for the basal medium was established, using the Hellige comparator and appropriate indicators of the sulphonphthalein series. To 500 ml. quantities of the broth were then added the calculated amounts of 5*N* HCl or 5*N* NaOH to give *p*H values from 3 to 10 at intervals of 0·5. The medium in each flask was then buffered by adding appropriate amounts of buffer mixtures, and 5% of 0·04% solution of indicator added. The volume in

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each flask was finally adjusted to 600 ml. The buffers and indicators used to cover the range from *p*H 3 to 10 are given in Table I.

The medium was distributed in test-tubes with Durham tubes in 10 ml. quantities. Sterilization was effected by three steamings of 30 min. each for media of *p*H 6·0–8·0, and two steamings of 30 min. for media of *p*H 3·0–5·5 and 8·0–10·0. The inoculum was 0·1 ml. of 1 : 100 dilution of a freshly clotted milk culture. Those strains not growing well in plain milk (9) were grown in suitably reinforced milk media. The cultures were incubated for 1 month at suitable temperatures and observations of turbidity, flocculation, sedimentation, change of *p*H and gas production made after 1, 2, 4, 7, 10, 14, 21 and 30 days.

Table I. *Indicators and buffers used in Exp. I*

*p*H	Indicator	Buffer solutions	Volumes in 600 ml. medium
3·0	Thymol blue	10 % citric acid, 10 % potassium citrate	38·5 ml. citric acid, 11·5 ml. potassium citrate
3·5	Brom-phenol blue	„ „	32 ml. „ 18 ml. „
4·0	Brom-phenol blue	„ „	26 ml. „ 24 ml. „
4·5	Brom-cresol green	„ „	20·5 ml. „ 29·5 ml. „
5·0	Brom-cresol green	„ „	15 ml. „ 35 ml. „
5·5	Chlor-phenol red	„ „	9·5 ml. „ 40·5 ml. „
6·0	Chlor-phenol red	„ „	5 ml. „ 45 ml. „
7·0	Brom-cresol purple	7·13 % $Na_2HPO_4.2H_2O$, 5·44 % KH_2PO_4	30 ml. Na_2H, 20 ml. KH_2
7·5	Brom-thymol blue	„ „	41·5 ml. „ 8·5 ml. „
8·0	Phenol red	„ „	47·5 ml. „ 2·5 ml. „
8·5	Phenol red	7·51 % glycine, 5·85 % NaCl, 1·0 *N* NaOH	45·5 ml. glycine + NaCl, 4·5 ml. NaOH
9·0	Thymol blue	„	40 ml. „ 10 ml. „
9·5	Thymol blue	„	32·5 ml. „ 17 ml. „
10·0	Thymol blue	„	28 ml. „ 22 ml. „

Exp. II. In this experiment the *p*H adjustments were carried out after sterilization, and tubing was necessarily carried out aseptically so that Durham tubes could not be used. As neither buffer mixtures nor indicators were added it was possible to measure the hydrogen ion concentrations of the cultures colorimetrically after incubation.

After adjustment of the bulk to *p*H 6·8, 500 ml. quantities of double digest casein broth plus 5 % yeast autolysate and 0·25 % phosphate were sterilized by autoclaving at 120° for 20 min. To each was then added aseptically 25 ml. of a 37 % aqueous solution of dextrose which had been sterilized by autoclaving at 120° for 15 min. One flask of medium was then used to establish a buffer curve by adding increasing amounts of *N* acid or *N* alkali to 50 ml. quantities and using the glass electrode for *p*H determinations. Calculated quantities of sterile 5 *N* acid or 5 *N* alkali were then added to the other batches of media to adjust these media to *p*H values from 3·5 to 5·0 and 8·5 to 10·5 at intervals of 0·5. The media were then tubed aseptically and the tubes packed in tins. Four open Petri dishes containing soda lime were placed on the tubes in each tin. After incubation for 36 hr. to detect contamination, the tubes were inoculated as before, and replaced in the tins. Observations for turbidity, flocculation and sedimentation were made, and after 30 days the *p*H of each tube of medium was determined with the Hellige comparator. The indicators used were methyl orange, brom-cresol green, brom-cresol purple, cresol red and cresol phthalein. The colorimetric and glass electrode *p*H values obtained with

this medium were compared and it was found that values agreed within 0·15 of a *p*H unit.

In both experiments the *p*H values of the media were determined with the glass electrode immediately before inoculation, and it can be seen from Table IV that, even when sealed tins and soda lime were used, sufficient absorption of carbon dioxide or alteration of the constituents of the media took place to lower the *p*H at *p*H 10·5 by over 1 unit.

Temperatures of incubation. The *Str. III*[1] strains and the *pastorianus* and *gracile* subdivisions of *L. III* were incubated at 22°. *Str. I.* and *L. I* and the remaining *L. III* strains were incubated at 28°. *Str. II* and *L. II* strains were incubated at 37°.

Cultures used

The strains of streptococci and lactobacilli used in these experiments were from the collection of one of us (J. G. D.), assembled from type culture collections and by donation from recognized workers on these groups of organisms. Each strain was under constant study, and all those for which data are given were true to type. The names used are those under which they were received.

Discussion of Tables II and IV

The data show very clearly the alkali tolerance and lack of acid tolerance of the streptococci and the acid tolerance and lack of alkali tolerance of the lactobacilli. Thus practically all the streptococci of groups I and II grew at *p*H 8·8, and of these only the enterococci grew well at *p*H 4·8. Very few rod forms grew at *p*H 8·8, but most of them were able to grow at *p*H 4 given favourable conditions.

Of the streptococci, group I ceased to grow below about *p*H 5, group II(*a*) (pyogenes, etc.) below *p*H 5·5–6, group II(*b*) ("*viridans*") below about *p*H 5·5, while group II(*c*) (enterococci) grew well at *p*H 5. All the group III streptococci grew at *p*H 4·4 and three of five strains (excluding *Str. diaceti-lactis*) grew at *p*H 4·1. Of the remaining streptococci only *Str. faecalis* grew at *p*H 4·4.

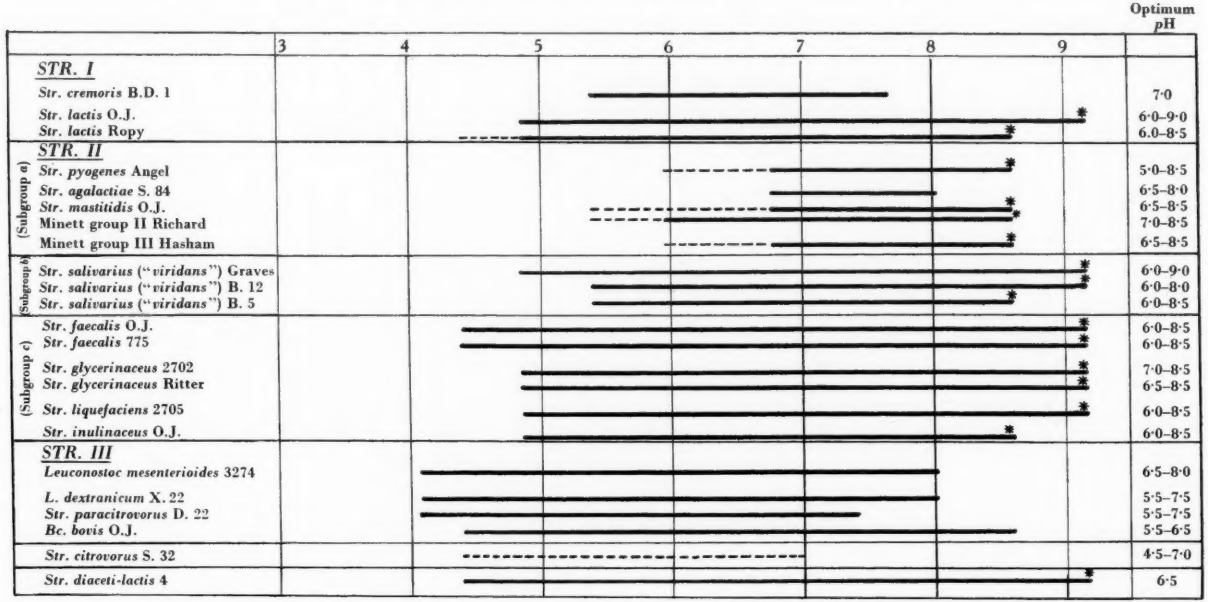
The most striking features of the data for the lactobacilli are the marked acid tolerance and lack of alkali tolerance of *L. bulgaricus*, the wide range of *L. pentoaceticus* and the rather narrow range of the *L. pastorianus* group.

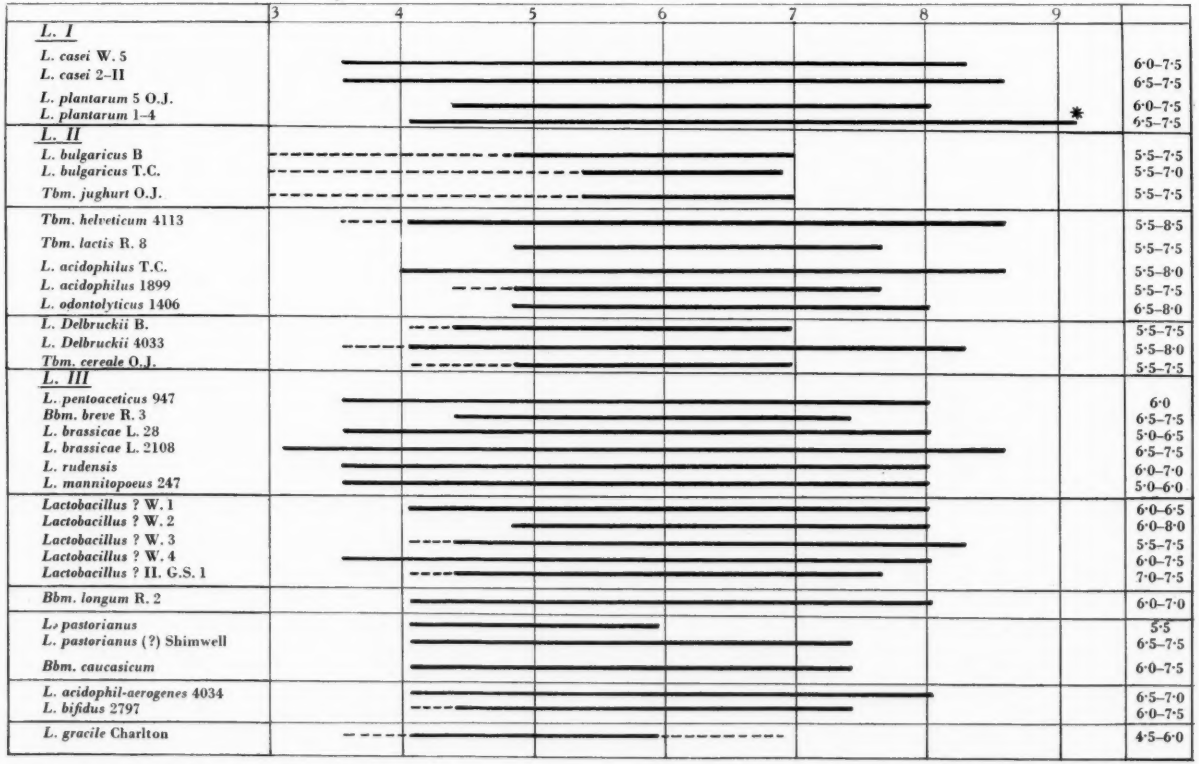
*Optimum p*H *for growth*

The optimum *p*H for growth was based on the rate of appearance of visible growth as indicated by turbidity and sediment. These values are given in Table II. Generally speaking, the optimum initial *p*H for growth is towards the alkaline end of the range for *Str. I* and *II*(*a*). *Str. II*(*b*) and (*c*) both have a broad optimum zone. *Str. III* has a fairly wide optimum zone rather towards the acid side of the neutral point.

[1] For classification see refs. (10), (11).

Table II. *The* pH *range and optimum* pH *for growth of streptococci and lactobacilli*





* Indicates that the organism grew at the highest *p*H used. A full line indicates normal growth: a dotted line indicates weak growth.

Table III. *Effect of* pH *on gas production*

Initial pH (glass electrode)	3·10	3·57	4·07	4·40	4·85	5·38	5·94	6·75	7·10	7·40	8·02	8·60	8·96	9·15	Optimum initial pH for gas production
Str. III															
Leuconostoc mesenterioides 3274	–	–	–	–	–	–	+	+ +	+ +	+ +	+ +	–	–	–	7·5
Str. paracitrovorus D. 22	–	–	+	+ +	+ +	+ + +	+ + +	tr	+	+	–	–	–	–	6·0
Leuconostoc dextranicum X. 22	–	–	+ +	+ + +	+ +	+ + +	+ + +	+ +	+ +	+ +	+ +	–	–	–	6·0
Bc. bovis O.J.	–	–	–	+ +	+	+ +	+ +	+	+	+	–	–	–	–	5·5–6·0
Str. diaceti-lactis 4	–	–	–	+	+	+	–	–	–	–	–	–	–	–	5·5
Str. citrovorus S. 32	–	–	–	–	+	+	+	+	+	–	–	–	–	–	5·5
L. III															
L. pentoaceticus 947	–	–	–	–	+ +	+ + +	+ + +	+ +	–	+	+	–	–	–	5·5–6·0
Bbm. breve R. 3	–	–	–	–	+	–	+	–	+	+	–	–	–	–	6·0
L. brassicae L. 28	–	–	tr	+	+ +	+ + +	+ + +	+ + +	+ +	+	+	–	–	–	5·5–6·0
,, L. 2108	–	–	–	–	–	–	tr	–	+	–	–	–	–	–	6·0
L. rudensis	–	–	–	+ +	+ +	+	+ + +	+ + +	+ +	+ +	+ +	–	–	–	6·0–7·5
L. mannitopoeus 247	–	–	–	+	–	+	–	–	+	+	+	–	–	–	6·0
Lactobacillus ? W. 1	–	–	tr	+ +	+ +	+ + +	+ +	+ +	+ +	+ +	+	–	–	–	5·5–7·0
,, ? W. 2	–	–	–	–	–	–	–	+	+	+ + +	+ +	–	–	–	7·5
,, ? W. 3	–	–	tr	–	+	+	–	–	–	–	–	–	–	–	5·5
,, ? W. 4	–	–	tr	–	–	+	–	–	–	–	–	–	–	–	5·5
Bbm. longum R. 2	–	–	+	–	+	+ + +	+ + +	+	+ +	–	–	–	–	–	6·0–6·5
L. pastorianus	–	–	–	+	–	+	+	–	–	–	–	–	–	–	5·5
,, (?) Shimwell	–	–	–	–	–	–	tr	–	+	tr	–	–	–	–	7·0
Bbm. caucasicum O.J.	–	–	–	–	–	–	–	+	–	–	–	–	–	–	6·5
L. bifidus 2797	–	–	–	–	–	+	+	+	+	+	–	–	–	–	5·5–7·0
L. acidophil-aerogenes 4034	–	–	–	tr	–	+	+ +	+ + +	+ +	+ +	+ +	–	–	–	6·5
L. gracile Charlton	–	+	+	+	+	+	+	–	–	–	–	–	–	–	4·0–6·0
Lactobacillus ? 11. G.S.1	–	–	+	+	+	–	+	+	+	+	–	–	–	–	6·0–7·5

Table IV. *Exp. II. Final* pH *values*

Initial *p*H (glass electrode) ...	3·54	4·04	4·42	4·86	8·32	8·80	9·27	9·85	10·54
Control (37°)	3·5	3·8	4·5	5·1	7·8	*c.*8·3	8·7	—	9·3
„ (28°)	—	4·1	—	5·2	7·8	8·3	8·7	9·1	—
Str. I									
Str. cremoris R.W.	3·5	4·0	4·2	5·4	**4·7**	8·3	8·4	9·1	9·3
„ J.	3·5	3·9	4·2	5·2	**4·7**	**4·6**	8·7	9·1	9·3
„ O.J.	3·5	3·9	4·3	5·2	7·8	—	8·7	9·1	9·3
„ H.P.	—	4·0	4·3	5·2	7·8	8·1	8·7	9·1	9·3
„ 18 O.J.	3·5	4·1	4·4	5·2	7·8	8·3	8·8	9·1	9·3
Str. lactis 2700	3·5	4·1	4·4	—	**4·7**	**4·7**	**4·7**	9·1	9·3
„ I.	3·6	4·1	4·4	5·2	**4·7**	**4·7**	**4·7**	9·1	9·3
Str. thermophilus 219	3·5	3·9	4·3	5·4	—	**5·7**	8·3	8·3	9·3
„ 542	3·4	4·0	4·1	**4·4**	**4·7**	**4·8**	8·3	8·9	9·7
Str. II									
Str. pyogenes Benfield	3·5	3·9	4·1	5·4	—	**5·2**	8·3	*c.*8·7	9·4
„ Azgaz	3·5	3·9	4·3	*c.*5·7	**5·6**	**5·6**	**5·3**	8·8	9·1
Str. agalactiae 3444	3·5	3·9	4·2	5·6	**5·5**	**5·2**	8·3	8·7	9·2
Minett group II, S. 32 (M)	3·5	3·9	4·1	5·5	**5·8**	**5·2**	8·3	8·7	9·1
„ Venus	3·5	3·9	4·1	5·3	**5·6**	**5·2**	**5·7**	8·7	9·1
Minett group III, M. 20	3·5	3·9	4·1	5·3	**5·2**	**5·2**	**5·2**	8·4	9·2
"Animal *pyogenes*" Radford C	3·5	3·9	4·2	5·5	**5·6**	*c.*5·2	8·3	8·7	9·1
„ S. 89	3·5	3·9	4·1	5·5	**5·3**	**5·6**	**5·6**	8·5	9·1
Str. salivarius B. 12 ("*viridans*")	3·5	3·9	4·2	4·9	**4·5**	**4·5**	**4·6**	—	**4·7**
„ 446	3·5	3·9	**3·6**	< **4·0**	**4·5**	*c.*7·0	8·3	8·6	9·1
Str. faecalis O.J.	3·5	3·9	4·4	**4·5**	**4·7**	**4·7**	**4·8**	**4·8**	**5·0**
„ 370	3·4	3·9	4·3	**4·7**	**5·5**	**5·4**	**5·4**	**5·4**	*c.*5·8
„ Z. 111	3·5	—	4·2	**4·2**	**4·3**	**4·2**	**4·4**	**4·2**	9·3
Str. glycerinaceus 2702	3·5	3·9	4·2	5·0	**4·7**	**4·7**	**4·7**	**4·7**	**4·7**
„ T. 65	3·5	4·0	4·1	**4·1**	**4·5**	**4·4**	**4·5**	**4·5**	**4·6**
Str. liquefaciens T. 63	3·4	—	4·1	**4·5**	**4·5**	**4·5**	**4·5**	**4·6**	*c.*5·0
Str. III									
Leuconostoc mesenterioides X. 5	3·5	4·1	4·4	**4·3**	7·8	—	8·7	9·1	9·2
Str. paracitrovorus D.N. 2	3·5	4·0	4·1	**4·7**	—	**5·5**	8·7	9·1	9·3
Str. citrovorus S. 146	3·6	4·1	*c.*4·4	5·2	7·8	8·3	8·7	9·1	9·2
„ (*Ped. viscosus*)	3·7	4·1	—	**4·1**	7·8	8·1	8·7	9·1	9·2
„ (*Ped. perniciosus*)	3·5	4·1	*c.*4·4	5·2	—	**5·6**	8·7	9·1	9·3
Str. diaceti-lactis 6	3·5	4·1	4·4	**4·7**	**4·7**	**4·7**	**4·7**	8·9	9·2
„ 15	3·5	4·1	—	**4·3**	**4·6**	**4·7**	*c.*8·6	9·1	9·3
L. I									
L. casei R. 12	—	**3·7**	**3·7**	**3·7**	**3·9**	**3·9**	*c.*8·7	9·1	9·3
L. plantarum 3 G.S. 2	3·5	**3·8**	**3·9**	**4·1**	7·8	—	8·6	9·1	9·2
„ 12 G.S. 1	3·5	**3·6**	**3·7**	**3·6**	**4·1**	**4·1**	8·6	9·1	9·3
„ A.M.	3·5	**3·4**	**3·4**	**3·5**	**4·0**	**4·1**	*c.*8·7	9·1	9·2
B. cucumeris	**3·2**	**3·3**	**3·4**	**3·5**	**3·8**	8·4	8·7	9·2	9·3
L. arabinosus F. 17–5	**3·1**	**3·3**	**3·5**	**3·5**	**3·9**	—	8·7	9·1	9·3
L. II									
L. bulgaricus B.	3·6	4·0	4·3	**4·1**	7·7	7·9	8·3	8·8	—
L. lactis-acidi B.	3·5	**3·2**	**3·3**	**3·3**	—	**4·1**	8·3	8·7	—
L. lactis Henneberg	3·5	**3·5**	**3·6**	**3·7**	7·7	7·9	8·3	—	—
L. acidophilus 1724	3·5	4·0	4·2	**4·0**	7·7	—	8·3	*c.*8·7	—
L. III									
L. pentoaceticus 4037	3·5	4·1	*c.*4·4	**4·3**	**4·7**	**4·7**	8·7	9·1	9·3
L. brassicae 4036	3·6	**3·8**	**4·0**	**4·2**	7·8	—	8·6	9·1	9·3
L. lycopersici 4223	3·5	3·9	3·9	**4·1**	7·8	8·3	8·7	9·2	9·4
L. mannitopoeus L–17	3·5	3·9	4·2	4·9	—	**4·8**	8·3	9·1	9·3
Bbm. longum R. 1 (37°)	3·5	3·9	**3·7**	**3·7**	7·7	8·6	—	8·9	9·5
„ R.I. (28°)	3·4	3·8	**3·7**	**4·1**	7·8	8·3	8·7	9·1	9·3
L. pastorianus	3·5	4·0	4·3	5·5	7·8	—	8·5	9·2	9·3
„ (?) Shimwell	3·5	**3·7**	**3·8**	**3·9**	7·8	8·3	8·8	9·1	9·3
L. ? K. 3	3·5	4·1	**4·1**	**4·3**	7·8	8·3	8·7	9·1	9·3

Bold type = cultures showing growth.
Light type = cultures in which no appreciable growth took place.

Of the rod forms *L. I* has a narrow optimum of about *p*H 7, while *L. II* and *L. III* resemble *Str. III* in having a fairly wide zone somewhat on the acid side.

Gas production

It is not claimed that the technique used (Durham fermentation tubes) permitted an accurate estimation of the amount of gas produced. It is probably safe to say that, under these conditions, production of gas above a certain minimum would be detected. The minimum naturally varies with the rate of production, since absorption is constantly taking place and even on the acid side loss to the air from a saturated solution would be considerable. Nevertheless with this technique no strains of group III (streptococci and rods) failed to produce gas, and only one strain in the other groups produced a trace of gas.

As might be expected, gas production was least at the extremes of the range of growth (Table III). The apparent optimum initial *p*H for gas production was usually the same as for growth. However, since gas was not usually detected until after growth had taken place (i.e. after the *p*H of the medium had fallen) and the alkaline media would absorb much of the CO_2 evolved, little significance can be attached to these figures.

*Differentiation of species by growth at given p*H *values*

Streptococci. The extreme alkaline tolerance of the enterococci has long been recognized (12) and growth at *p*H 9·6 has been used by Sherman & Stark (3) as a criterion for the identification of these streptococci. Thus these workers claim that this property differentiates *Str. faecalis* from *Str. lactis*. Our own findings with type cultures confirm the results of these authors. The differentiation of *Str. cremoris* from *Str. lactis* by failure to grow at *p*H 9·2 (4) has also been confirmed by us, but with the observation that three out of six strains of *Str. cremoris* failed to grow at *p*H 8·3. The frequent failures of *Str. cremoris* to grow without milk is a characteristic that must be borne in mind when carrying out such tests, and is in fact one of the most reliable methods of differentiating *Str. cremoris* from *Str. lactis*.

Lactobacilli. No attempts to differentiate lactobacilli by this method appear to have been made. The great significance of other factors such as the medium, oxygen tension and surface effects makes any such attempt very difficult.

In general the lower *p*H limits of growth for both streptococci and lactobacilli run parallel to the final *p*H values given in the literature (3, 4).

Summary

The *p*H ranges of growth of streptococci and lactobacilli in dextrose yeast casein digest broth have been studied. Nearly all the group I and II streptococci grew at *p*H 8·8, but only the enterococci grew at *p*H 4·8; all group III types (heterofermentative) grew at *p*H 4·4. Only a few lactobacilli grew at

*p*H 8·8 but most were able to grow at *p*H 4 if other conditions were favourable. The enterococci, *Str. lactis* and *Str. cremoris* could be differentiated by the alkaline limits of growth.

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239. EXAMPLES OF VARIATION WITHIN PURE CULTURES OF *STREPTOCOCCUS CREMORIS*

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Numerous instances of bacterial variation have been described. In many species of pathogenic organisms, colony variation has been correlated with changes in biochemical properties, serological differences and alterations in virulence. On the other hand observations of variation among saprophytic organisms are not so abundant. Orla Jensen(1) encountered several variable qualities of the lactic acid bacteria. Variation in *Str. lactis* in its response to air supply and also in its capacity to produce ropiness in milk was described by Hammer(2,3), and Harriman & Hammer(4) obtained from a pure culture of *Str. lactis* certain strains which differed in their rates of coagulating milk. Davis(5,6) described media suitable for the growth of milk bacteria in an attenuated condition and noted the "weakened" condition of certain bacteria isolated from cheese. These "weakened" strains fermented dextrose readily but attacked lactose only slowly. Abnormal types of *Str. cremoris* which formed chains of giant cells were also reported. Okulitch & Eagles(7) found that the propagation of a strain of *Str. cremoris* in certain sugars with casein digest broth as the nitrogen source caused the organisms to lose the power of utilizing lactose in milk. Substrains of *Str. cremoris* and *Str. lactis* varying in their ability to attack maltose and sucrose were described by Sherman & Hussong(8), while Yawger & Sherman(9) reported the isolation from samples of milk of variants of *Str. lactis* which did not ferment lactose.

Indications of variability in certain pure cultures of lactic streptococci form the basis of this paper. The strains studied were those isolated at the Institute and used extensively for cheesemaking in New Zealand. They were strains of *Str. cremoris* (Orla Jensen), selected on the basis of ability to withstand "cooking" temperatures in the vicinity of 100° F. and thus to produce acid steadily throughout the manufacturing process. They were purified frequently by plating and during this procedure it was noticed that some rapid acid-producing strains gave only a few colonies which reached the activity of the parent culture. Investigation of this phenomenon led to the isolation of the variants reported below.

A. Variation as reflected in form of colony

One pure strain (HP) when streaked on the surface of the solid substrate gave rise at times to colonies which varied from the "normal". In acid-producing streptococci such as those used in cheesemaking, the colony which is

capable of causing rapid coagulation of milk is taken to be the normal type. Grown at 22° C. on whey agar containing yeast extract, it is generally a smooth cream convex opaque colony about 0·5 mm. in diameter and when transferred to tubes of sterilized milk it possesses the power of clotting the milk at 22° C. within 24 hr. The culture thus produced retains this clotting power apparently indefinitely. Since the single strain cultures used as cheese starters must be active acid producers, the normal colony is the type selected during purification and consequently such a colony was the starting point of the culture studied. It had been purified by plating more than twenty times in the course of three years.

Sugar fermentation tests confirmed the classification as a species of *Str. cremoris* (Orla Jensen). Milk was coagulated in 18 hr. at 22° C. Vitality tests on the culture (10) always gave quite high figures, these results being borne out by performances in the cheese vat. When a freshly inoculated milk culture was held at a temperature of 37° C. for 6–7 hr., examination under the microscope showed medium chains of normal cocci with the terminal coccus characteristically swollen. The organisms were completely lysed by a bacteriophage which so far has proved strain specific.

The strain was transferred daily in milk. When streaked on yeast whey agar it appeared to be a homogeneous culture, giving colonies which were predominantly of the normal type; any smaller or divergent colony being so much in the minority that it was regarded as being due to overcrowding.

Isolation of slow clotting type

A more careful examination of plates very thinly sown revealed the fact that, at times, there existed different colony types:

(1) The normal one day clotter.

(2) An umbonate colony of similar diameter but consisting essentially of a creamish convex smooth centre and a flat grey margin which often contained minute papillae. This type was found to be a slow grower, requiring 2–4 days to bring about clotting and therefore of no use for starter work.

In the absence of means of controlling accurately the composition of the yeast whey agar, reliable data concerning the conditions under which the production of these colony types was favoured could not be obtained, but incubation of plates at temperatures above 20° C. seemed to favour the appearance of the umbonate slow clotting type. Rapid and slow growing forms were present in variable proportions in milk cultures derived from both types of colony. On solid media the colonies gave more or less true breeding with "intermediates". Endeavours to pick from the centre and margin of the umbonate colonies did not yield clear cut results but, in general, continued transfers from the edge gave colonies predominantly of the umbonate type, while transfers from the centre resulted in the production of a mixture of the umbonate and normal.

In deep agar the two types were represented by a cream round colony with

entire edge and a cream lobate colony. The organisms were identical morphologically, exhibiting the typical appearance of the original culture when grown in milk at 37° C. for 6 hr. The only difference under these conditions was the less prolific growth of the slow clotting culture. They were both acted upon by the specific bacteriophage.

Isolation of type which failed to ferment lactose

During trials of different growth conditions in which the composition of the nutrient substrate was altered and the incubation temperature held constant at 22° C., a further variant was obtained. Streaked on a lactose agar medium, the same strain of *Str. cremoris* gave, besides the two described, a growth of conglomerate colonies which when transferred to milk were mostly one day clotters. These colonies split into two distinct colony types on yeast whey agar:

(*a*) The normal cream convex colony.

(*b*) A flat greyish type which had lost the power of producing acid from lactose. In milk the acidity produced in 14 days at 22° C. was less than 0·04% calculated as lactic acid. The colonies, however, readily coagulated milk to which glucose had been added (average acidity produced = 0·85%); therefore they cannot be regarded solely as "weakened" strains. A few colonies were found to produce about 0·2% of acid in milk in 14 days but generally the flat variant retained its characteristics after twenty transfers on solid media. When transferred in glucose milk the variant remained active but did not regain the power of utilizing lactose. Streaked on yeast whey agar containing glucose, the flat colonies or cultures derived from them gave colonies of the "normal" appearance (cream convex) but these still failed to produce acid in milk. Sugar reactions of the normal and variant in 0·5% nitrogen casein digest broth were similar with the exception of lactose, which the variant failed to ferment (Table I). The variant was still susceptible to the specific phage and showed the same typical appearance at 37° as the parent culture.

Table I. *Acidity in parts per thousand as lactic acid*

	Levulose	Glucose	Mannose	Galactose	Lactose
Normal	4·0	4·5	4·0	2·0	4·0
Variant	4·5	5·0	4·3	1·0	0·0

The occurrence of the degenerate conglomerate colonies was very spasmodic and up to the present all attempts to define the precise conditions under which dissociation occurs have been unsuccessful. Many attempts resulted in the production of 100% of this type but even then the actual factor or factors responsible for this marked change could not be determined.

B. Variation resulting in the production of ropiness in milk

The production of slime or ropiness in milk is a well-known characteristic of certain strains of *Str. cremoris*. It seems to develop more readily when the cultures are grown at low temperatures. Two separate strains (RW 1) and (K)

which were subcultured daily with a platinum loop into milk at 22° C. in the laboratory, suddenly acquired the property of forming slime. The strains were distinguishable morphologically after 6 hr. growth in milk at 37° C. and each was susceptible to its own specific phage. When the strains were streaked on yeast whey agar and colonies picked into milk, it was found that only a certain proportion of the cultures gave slime, the others being apparently normal. In both strains this was the only variable character, there being no difference in colony type, morphology, phage susceptibility or acid production. As has been reported by other workers, the slimy cultures were found to lose their slimy character when they were transferred daily at higher temperatures, but regained it when returned to the 22° C. incubator. During the period of investigation most of the non-slimy cultures derived from these two strains began to form slime after about twenty transfers at 22° C., except when a massive inoculum was used in the transference of the culture from day to day. The use of 0·1–0·2 ml. as an inoculum to 10 ml. milk seemed to prevent the acquirement of a slimy character. The ropiness of these cultures did not influence their usefulness as cheese starters, the performances of the ropy and non-ropy cultures in the vat being similar.

C. Variations in acid-producing activity

The vitality test technique(10), carried out with due regard to aseptic precautions to avoid phage contamination, gives a method of determining the relative rates of acid production of different starter cultures under conditions somewhat similar to those obtaining in the cheese vat. From some stock strains of *Str. cremoris* it was possible to obtain relatively stable rapid and slow acid producing substrains in which no other difference was discernible. Both were susceptible to the particular strain specific bacteriophage.

D. Variation in ability to withstand high temperature conditions

A difference among individuals of a pure line culture to withstand a temperature of 37° C. as judged by morphology and vitality tests was observed during work with a further strain of *Str. cremoris* (R_1). This was a rapid acid former when freshly purified but it gradually lost some of this power on daily transfer in milk at 22° C. Cultures after treatment at 37° C. consisted of long chains of normal cocci showing no signs of involution forms, but later, after several transfers at 22° C., a retest at 37° C. in milk showed the presence of chains in which the cocci were distinctly involuted, being swollen and elongated in appearance. Coincidently the organisms weakened in performance in the cheese vat. Colonies, although normal in appearance, gave rise to two different substrains:

(1) An active acid producer in the cheese vat, showing no involution forms at 37° C.

(2) A culture of poor vitality, badly involuted at 37° C.

Both these substrains coagulated milk readily at 22° C., acidities after 18 hr. being in the vicinity of 0·8 and 0·68% lactic acid respectively. The difference in vitality was well marked in the cheese vat, culture (2) being particularly slow after the whey was run off. The bacteriophage active against this *Str. cremoris* strain was specific in action and lysed the two substrains readily.

E. Variation of individuals within in a culture to phage attack

The problem of quantitatively estimating differences in resistance of organisms to the action of a race of bacteriophage is complicated by the fact that in some instances the results vary according to whether a solid or liquid medium is employed. Probably the most satisfactory method for the lactic streptococci and their phages is that of preparing a series of dilutions of the phage and placing a loopful of each on a solid agar medium thickly spread just previously with the susceptible organism. On incubation, clear areas of lysis are obtained with the stronger concentrations of phage and a graduated number of plaques with the weaker concentrations. Observations of phage action upon single colonies and cultures derived from them showed that there sometimes exist in a pure culture individuals which are acted upon more readily than others by a specific phage. It was possible to propagate phage on the different types, and when this was done there was apparently no difference between the phages so obtained. From pure cultures some colonies (or cultures) gave titres up to 10^{-6}, while others showed merely a few plaques on the areas containing the neat phage. No completely resistant colony was found.

In the *Str. cremoris* strain (R_1), described above as containing individuals showing different morphology after cultivation at 37° C., an interesting phenomenon regarding phage action was observed. A specific phage, while possessing the power of multiplying on the constituent cells of the culture, frequently failed to cause their complete dissolution before coagulation of the milk had taken place. On plates, the lytic areas were "foggy" and not clear cut as is generally the case after 10–20 hr. incubation at 30° C., but on further incubation the areas cleared completely. Of the two substrains isolated from the culture, although both were completely lysed in milk, one gave clear cut results on plates, the other "foggy" areas clearing later. It would appear, therefore, that there existed in a pure culture individuals on which the phage acted more readily than on others, the fogginess produced being due to the longer time required for the disappearance of the more resistant forms.

A further instance illustrating the fact that organisms within a pure culture may vary in their response to phage action has already been recorded (11). A race of phage active against one pure strain (K) showed no evidence of lysis either in liquid or on solid media when tested against another closely related but distinct strain (RW). Purification of the non-susceptible organism (RW) by plating gave rise to a culture which was not susceptible to the phage in milk or broth but allowed the formation of plaques on a solid medium. Further

plating after several subcultures in milk, finally gave a culture which was readily attacked by the phage in both types of media. The balance within the pure culture was thus gradually changed over the period of investigation. The difference in resistance of the RW substrains to this particular race of phage was the only detectable change. The characteristic morphology (after treatment at 37° C.) and the rate of acid production which permitted a separation of the original strains K and RW remained the same.

DISCUSSION

The results provide further illustration of the instability of "pure" cultures of lactic streptococci which would ordinarily be regarded as being constant in properties. In the lactic acid bacteria, the property which is usually of most importance is the power of producing acid from lactose in milk. Assuming the changes in colony growth on the surface of the solid medium to be a true reflexion of the condition of the organisms in milk, there is, in a presumably normal pure culture, a fluctuation of individuals possessing varying degrees of acid-producing power. The rapid daily transfers in milk, while giving ample opportunity for normally active individuals to dissociate, would "dilute" out the few dissociated cells or slower growing variants, thus enabling the culture as a whole to maintain its original characteristics. Occasionally the balance appeared to be unstable, since in some cultures derived from normal colonies the different types of individuals could be demonstrated more readily than in others.

Particular interest is attached to the fact that a specific phage was available for each strain in which variability was studied. The facts that the variants were morphologically similar to the parent culture and that they were lysed by the same specific phage rendered it extremely unlikely that they were merely contaminants—a possibility which is always a pitfall in work dealing with bacterial variation. The substrain which failed to ferment lactose was lysed by the specific phage for the strain of *Str. cremoris* in question. Thus the enzyme-forming complex of the organism is apparently independent of the complex prominent in phage action.

The importance of recognizing the existence of variants such as those which have lost the power of elaborating the enzyme lactose is emphasized in taxonomic problems. Sherman and his co-workers (8, 9, 12) have drawn attention to variability among substrains derived from pure cultures, especially in relation to their power of attacking carbohydrates. The substrain described above was identical with *Str. cremoris* in all respects except that it did not ferment lactose. The change in sugar fermentation in this variant of known origin did not affect the morphology at 37° C. or the susceptibility to the specific phage.

SUMMARY

Indications of variability within pure strains of *Str. cremoris* have been studied.

In one strain changes in acid-producing power were made evident by changes in colony form. A variant which failed to ferment lactose but was still susceptible to the specific race of bacteriophage and exhibited the same morphology (after treatment at 37° C.) as the parent culture is described.

It was found that variation was well marked within some strains, especially in regard to acid production (or vitality), response to high temperature conditions, power to produce ropiness in milk, and degree of resistance to phage attack.

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240. THE EFFECT OF LIGHT ON THE VITAMIN C OF MILK IN DIFFERENT CONTAINERS

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(With 2 Figures)

It has been shown by Mattick & Kon (1) and by Kon & Watson (2) that exposure of milk to light in the usual clear glass bottles leads to rapid reversible oxidation and consequent destruction of vitamin C. In recent years a different type of container, the wax impregnated carton, has been increasingly used for the retail distribution of milk. The carton is translucent and transmits some light; much less, however, than a clear glass bottle. It was of interest, therefore, to determine to what extent milk placed in such containers would be affected by light. The destructive effect of visible light diminishes rapidly from the blue end of the spectrum to red which is inactive (2). For this reason two types of coloured bottles used commercially, i.e. a green and a brown beer bottle, were also tested.

Milk was obtained directly from the udder in the absence of light and was placed in cartons, coloured bottles, clear glass bottles, clear glass bottles wrapped in one layer of no. 300 red Cellophane and in clear glass bottles enclosed in a box and completely protected from light. These samples were then placed outside in a shady place. The coloured bottles were of 1 pint, the other containers of $\frac{1}{2}$ pint capacity. Separate containers were removed at intervals and analysed for reduced and total ascorbic acid as described by Kon & Watson (2).

The results of a typical run are presented in graph form, Figs. 1 and 2, and data for two runs are given in Tables I and II.

The results show, as would be expected, that the destruction of vitamin C was less severe in cartons than in clear glass. After the first hour of exposure

Table I. *Effect on the vitamin C of milk of exposure to light in different containers of $\frac{1}{2}$ pint capacity*

R, reduced; T, total, ascorbic acid in mg./100 ml. Original milk: R = 2·00; T = 2·15 mg./100 ml.

Time of exposure hours	Clear glass bottle		Carton		Clear glass bottle wrapped in red Cellophane	
	R	T	R	T	R	T
½	1·09	1·59	1·55	1·81	1·89	2·00
1	0·53	0·83	1·25	1·59	1·70	1·96
2	0·0	0·68	1·06	1·38	1·66	1·85
4	0·0	0·53	0·91	1·06	1·56	1·74
5	0·0	0·60	0·75	0·98	1·51	1·59
6	0·0	0·53	0·57	0·98	1·49	1·51

there was roughly twice as much total vitamin C in the former container as in the latter. The brown bottles were remarkably protective, the green bottles less so, in agreement with previous observations concerning the activity of different wave-lengths (2). The latter were, nevertheless, slightly superior to the cartons.

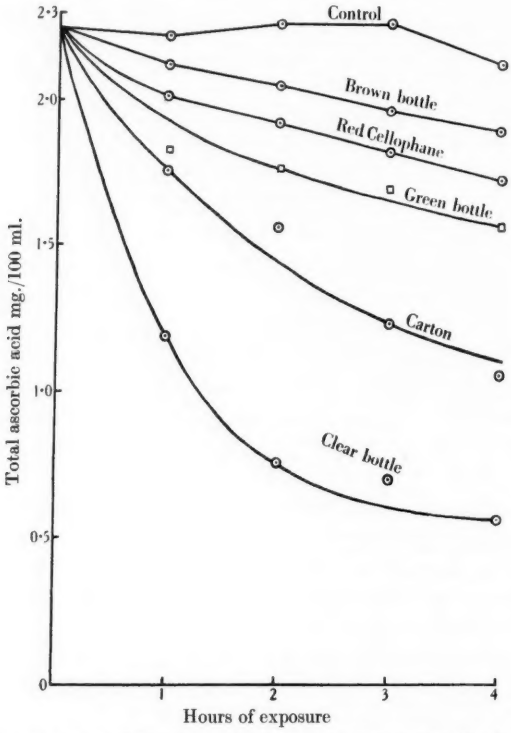

Fig. 1. Effect of exposure of milk in different containers to light on its content of total ascorbic acid.

Table II. *Effect on the vitamin C of milk of exposure to light in different containers*

R, reduced; T, total, ascorbic acid in mg./100 ml. Original milk R = 2·28; T = 2·25 mg./100 ml.

Time of exposure hr.	Clear glass bottle*		Carton*		Green bottle†		Clear bottle wrapped in red Cellophane*		Brown bottle†		Control*	
	R	T	R	T	R	T	R	T	R	T	R	T
1	0·70	1·19	1·34	1·76	1·74	1·82	1·91	2·02	2·04	2·12	2·11	2·22
2	0·0	0·76	1·07	1·56	1·54	1·76	1·78	1·92	1·88	2·05	2·04	2·26
3	0·0	0·70	0·77	1·23	1·41	1·69	1·74	1·82	1·77	1·96	2·01	2·26
4	0·0	0·56	0·60	1·06	1·34	1·56	1·64	1·72	1·68	1·89	2·01	2·12
‡7(21)	0·0	0·53	0·51	0·96	0·97	1·33	1·47	1·62	1·61	1·92	1·94	1·99
‡13(27)	0·0	0·46	0·23	0·93	0·47	1·19	1·37	1·56	1·47	1·86	1·71	1·81

* Half-pint capacity. † One pint capacity.

‡ Bottles left outside overnight. 14 hr. from sunset to sunrise. Sharp frost during night.

Several more runs were carried out in which milk was exposed to light in the different containers for fixed periods of time and was then kept in darkness before sampling. This was done to imitate practical conditions where milk is often exposed to light on the doorstep and then kept for some time in a larder or cupboard. The results were very similar to those reported above and the

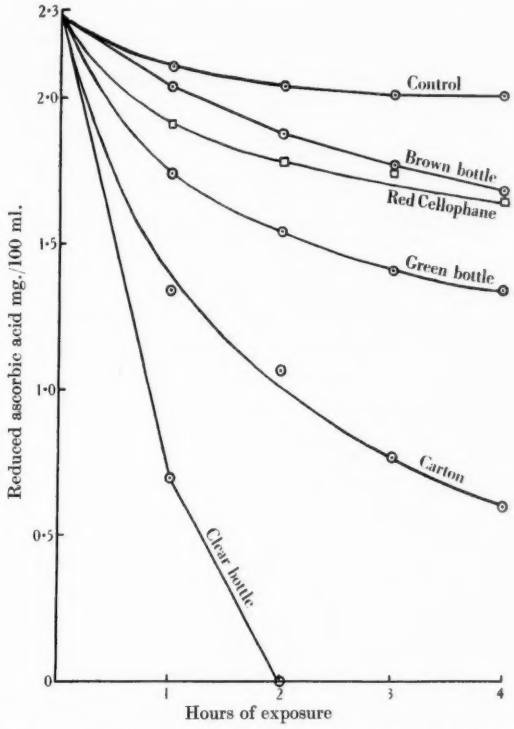

Fig. 2. Effect of exposure of milk in different containers to light on its content of reduced ascorbic acid.

Table III. *Effect on the vitamin C of milk of exposure to light and subsequent storage in darkness in different containers of $\frac{1}{2}$ pint capacity*

Reduced ascorbic acid in mg./100 ml. Original milk 2·16 mg./100 ml.

Exposure to light hr.	Storage in darkness hr.	Type of container: Clear bottle	Carton	Green bottle	Clear bottle wrapped in red Cello-phane	Brown bottle	Control
1	0	0·62	1·60	1·87	1·89	2·04	2·14
	1	0·52	1·35	1·77	2·03	1·95	2·11
	3	0·56	1·70	1·75	1·63	1·99	2·06
	6	0·54	1·15	1·67	1·82	1·86	1·95
3	0	0·28	1·46	1·68	1·89	1·97	2·06
	1	0·23	1·30	1·30	1·87	1·99	1·98
	3	0·11	1·10	1·14	1·88	1·85	1·93
	6	0·06	1·15	1·19	1·72	1·87	2·04

same order of efficiency of the containers was maintained. For the sake of brevity only the results of measurements of reduced ascorbic acid in a typical run are given in Table III.

SUMMARY

The destruction of vitamin C in milk under the action of light was studied in different containers. Brown glass bottles showed little destruction, green glass bottles considerably more, and wax impregnated cartons still more. Nevertheless, in the latter, twice as much vitamin C survived as in clear glass bottles.

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241. KAY AND GRAHAM'S PHOSPHATASE TEST

(A) MODIFICATIONS IN TECHNIQUE
(B) EFFECT OF BACTERIAL GROWTH

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KAY & GRAHAM'S phosphatase test(1), first described in 1935, has during the last four years been thoroughly examined both in this country and in several countries abroad, and has proved itself a convenient and reliable method for the control of the efficiency either of holder or of high-temperature short-time pasteurization(2,3,4,5,6,12).

The purposes of the present paper are (i) to describe a modification in the technique of the test which saves one operation, and thus both time and material, without any serious loss of precision, (ii) to draw attention to certain minor but important precautions which considerable experience of the test has shown to be necessary, and (iii) to record the effect on the test of bacterial activity.

THE USE OF SODIUM HEXAMETAPHOSPHATE TO PREVENT PRECIPITATION AFTER ADDING ALKALI

In the original description of the test, the operations subsequent to adding Folin and Ciocalteu's reagent consisted in adding 2 ml. of sodium carbonate to 10 ml. of filtrate, in a test-tube, mixing the whole and placing in a boiling water bath for 5 min. *and again filtering.* It has since been found that the addition of sodium hexametaphosphate, before placing the tubes in the boiling water bath, prevents the precipitation which normally occurs on boiling and dispenses with the second filtration.

The sodium hexametaphosphate is most conveniently added to Folin and Ciocalteu's reagent,[1] which instead of being diluted with twice its volume of distilled water is now diluted with twice its volume of a 5%[2] sodium hexametaphosphate solution (made by dissolving the salt[3] in warm distilled water and cooling).

On adding the Folin's reagent to the hexametaphosphate solution there should be no effervescence. This may be avoided by sifting the flakes to remove the efflorescent white powder (probably $NaHCO_3$). The salt should be kept in a tightly stoppered bottle.

[1] Sodium hexametaphosphate cannot with advantage be added to the sodium carbonate solution, as it was found to be much less stable in presence of this concentration of sodium carbonate.

[2] In this paper, "per cent" indicates grams present in 100 ml. of solution.

[3] The salt used was obtained from British Drug Houses, Ltd.; other brands have not been tested.

After adding the sodium hexametaphosphate solution to Folin and Ciocalteu's reagent, the solution remains stable for the same length of time as the diluted Folin's reagent itself, i.e. not less than 3 weeks at ordinary room temperature in the south of England.

Table I. *The effect of using various concentrations of sodium hexametaphosphate on the phosphatase test (test "B")*

Dilute Folin's* reagent containing	Lovibond blue units						
	Pasteurized milk		Milk heated 146° F. for 20 min.		Pasteurized milk +0·3% raw		After boiling
No $(NaPO_3)_6$	1·4	1·5	2·2	2·4	4·7	4·8	Filtered hot
1% ,,	1·5	1·6	2·2	2·2	4·7	4·9	Slightly cloudy (not filtered)
1·5% ,,	1·4	1·5	—	—	4·7	4·9	Do.
2% ,,	1·4	1·5	—	—	4·7	4·9	Clear
5% ,,	1·5	1·5	—	—	4·6	5·0	Do.
10% ,,	—	—	2·2	2·3	5·0	5·2	Do.

* The dilute Folin's reagent containing the sodium hexametaphosphate is prepared as described in the text.

The effect of heating, in a boiling water bath, on the development of the colour (when using sodium hexametaphosphate)

In the test originally described the first filtrate, after the addition of the sodium carbonate solution, was placed in boiling water for 5 min. It was found, however, that in the presence of sodium hexametaphosphate irregular results were obtained with some milks when the test tubes were left in boiling water for 5 min., and very occasionally when left for 3 min. In such cases there was an increase in blue colour, but the excess colour was found to be unstable.

Table II illustrates the magnitude of the differences found, and also shows the instability of the excess colour produced when the heating has been prolonged. In this table, columns (1) and (2) are readings of duplicate tests, determined within an hour, and columns (3) and (4) readings of the same solutions 18 hr. later (at room temperature). The excess colour disappears in from 15 min. to 24 hr.

Table III gives typical results obtained on comparing the original method with the new technique, using sodium metaphosphate, heating for 3 min. only, and omitting filtering.

In view of the results of a number of tests similar to those in Table II, it was decided to adopt, instead of 5 min., a heating time of *2 min.* in boiling water.[1] With this period of heating, the colour produced agrees closely (usually within the experimental error of $\pm 0{\cdot}2$ Lovibond blue units) with that obtained with the original technique, and is also stable for 24 hr.

[1] The water, which should be level with the solution in the tubes, is kept boiling, but not so violently as to cause the water to splash up into the tubes.

Table II. *The effect of heat on the development of blue colour in test "B" when using Folin's reagent containing* $(NaPO_3)_6$

Technique	Min. in boiling water	Lovibond blue units: Duplicates (1)	Duplicates (2)	Duplicates (3)	Duplicates (4)	
Original	5	18·9	20·1	18·9	20·0	
Metaphosphate	1	18·9	20·1	19·6	20·1	Mixed milk heated
,,	3¼	20·1	20·2	19·9	20·1	145° ± 0·1° F. for
,,	5½	25·2	27	20·1	20·2	10½ min.
,,	7	30+	30+	21	30+	
Original	5	6·7	6·9	6·6	6·9	
Metaphosphate	1	6·7	6·8	6·6	6·7	145° ± 0·1° F. for
,,	3¼	6·5	6·8	6·5	6·6	15 min.
,,	5½	8·1	9·0	6·2	6·7	
,,	7	9·0	9·2	6·2	6·3	
Original	5	2·8	2·8	2·7	2·9	
Metaphosphate	1	2·5	2·5	2·5	2·5	145° ± 0·1° F. for
,,	3¼	2·8	2·8	2·7	2·8	21 min. ± 15 sec.
,,	5½	3·1	3·5	2·4	2·5	
,,	7	3·6	3·6	2·4	2·5	
Original	5	2·1	2·2	2·1	2·2	
Metaphosphate	1	1·9	2·1	1·8	2·0	145° ± 0·1° F. for
,,	3¼	2·0	2·2	2·0	2·0	25 min. ± 15 sec.
,,	5½	2·0	2·2	1·8	1·9	
,,	7	2·1	2·2	1·8	2·0	
Original	5	3·5	3·6	3·4	3·6	Mixed milk heated 145° ± 0·1° F. for
Metaphosphate	2	3·4	3·4	3·3	3·4	20 min.
,,	4	3·3	3·4	3·3	3·4	
,,	5	3·4	3·7	3·1	3·2	
,,	6	4·0	4·4	3·0	3·1	
Original	5	7·1	7·3	7·1	7·4	
Metaphosphate	2	7·0	7·1	7·1	7·1	145° ± 0·1° F. for
,,	4	7·1	7·2	7·1	7·1	15 min.
,,	5	7·3	7·6	6·9	7·1	
,,	6	9·4	9·4	7·0	7·1	

Table III. *Some results obtained with commercial milk samples, using the original and modified technique (test "B")*

Milk sample no.	Original method no $(NaPO_3)_6$ filtered	New method with $(NaPO_3)_6$ not filtered	Milk sample no.	Original method no $(NaPO_3)_6$ filtered	New method with $(NaPO_3)_6$ not filtered
1	2·1	2·1	9	5·3	5·2
2	3·1	3·2	10	2·2	2·0
3	2·0	2·0	11	1·8	1·8
4	3·8	3·6	12	20·1	20·6
5	4·0	4·0	13	1·2	1·3
6	1·5	1·4	14	6·7	6·9
7	2·3	2·1	15	2·1	2·2
8	10·2	10·1	16	7·3	7·1

DIFFERENCES IN METHOD OF LABORATORY PASTEURIZATION

In this laboratory samples of milk are pasteurized in test-tubes totally immersed in a water bath, as described by Aschaffenburg and Neave in this journal. No differences in the results (using Kay & Graham's test "B") were found when pasteurization, using both 10 and 20 ml. quantities of milk per test-tube, was carried out in sealed tubes, stoppered tubes, and in stoppered

Table IV. *The original and modified techniques compared, using milk samples pasteurized in the laboratory. Also the effect of a less concentrated carbonate solution (from 52 tests)*

	Lovibond blue units		
	Original method	Using hexametaphosphate	
Milks	2 ml. 14% carbonate 5 min. in boiling water and filtered hot	2 ml. 14% carbonate 2 min. in boiling water	2 ml. 13% carbonate 2 min. in boiling water
(1) 145° ± 0·1° F., 30 min.	1·6	1·6	1·7
	1·8	1·7	1·8
(1) 145° ± 0·1° F., 20 min.	3·0	3·2	3·4
	3·2	3·2	3·5
(1) 144° ± 0·1° F., 25 min.	3·5	3·5	3·7
	3·6	3·6	3·9
(2) 144° ± 0·1° F., 30 min.	1·8	2·0	2·0
	1·9	2·1	2·1
(2) 144° ± 0·1° F., 25 min.	2·6	2·7	2·8
	2·9	2·7	2·8
(2) 144° ± 0·1° F., 15 min.	8·8	8·5	8·5
	8·9	8·7	9·1
(3) 145° ± 0·1° F., 20 min.	2·3	2·5	2·5
	2·4	2·5	2·6
(4) 145° ± 0·1° F., 20 min.	3·4	3·4	3·6
	3·5	3·5	3·7
(5) 145° ± 0·1° F., 20 min.	2·6	2·3	—
(6) 145° ± 0·1° F., 20 min.	2·7	2·5	—

The means of 74 results, using the original and modified methods, were respectively 2·85 and 2·84, with a maximum difference of 0·3 blue units between the two methods (single determinations).

tubes in which the pressure was released by removing the stoppers for a few seconds as soon as the temperature reached 145° F.

The test-tubes were made of the same glass, with a length of 150 mm. and an internal diameter of 15·5–16 mm.

Effect of carrying out the test in bright sunlight

Folin and Ciocalteu's reagent, at least under the conditions in which it is used in the phosphatase test, is not stable to bright sunlight coming through a glass window, though it is reasonably stable to diffuse daylight. The test therefore should not be carried out in sunlight. This is illustrated in Table V.

The effect of sunlight on milk phosphatase

A few tests have been made on raw milk and on milk containing some residual phosphatase without any noticeable effect of sunlight on the phosphatase activity. The milks were exposed in small test-tubes to bright sunlight for $2\frac{1}{2}$ hr.

Reagents

As stated by Kay & Graham, it is important that only the purest of reagents should be used. It is also advisable to test each new consignment of reagents before acceptance. Sodium diethyl barbiturate ("sodium veronal") has been

found, on occasions, to contain impurities which interfered very seriously with the test. In one case a blank on the reagent alone gave nearly 2·0 Lovibond blue units, as against the usual blank of 0·5 unit or less. The chloroform used for preserving the buffer-substrate solution should also be carefully tested, and in any case stored in a cool dark place.

Table V. *The effect of light on the colour development in the phosphatase test. The same milk sample handled under various conditions*

	Treatment prior to addition of sodium carbonate solution	Lovibond blue units Tests in triplicate (1)	(2)	(3)
Solutions containing Folin's reagent	Filtered* in subdued light. Tests completed immediately	1·1	1·2	1·2
,,	Filtered in ordinary light. Test completed immediately	1·2	1·2	1·2
,,	Filtered in brilliant sunlight. Tests completed immediately	4·0*a*	2·3*b*	1·9*c*
,,	Filtered in subdued light and left for 6 hr. before completing tests	1·1	1·2	1·2
,,	Filtered in subdued light and left for 6 hr. exposed to brilliant sunlight (through plate glass)	7·4	6·7	6·5
,,	Filtered by electric light and held at 15·5° C. overnight (14 hr.)	1·2	1·1	1·3
,,	Filtered by electric light and held at 28° C. overnight (14 hr.)	1·7	1·8	1·8

a = exposed for 23 min.; *b* = exposed for 15 min.; *c* = exposed for 12 min.

* Whatman no. 40 or 30, 9 cm. filter papers have been found satisfactory for both filtrations. No. 42, though satisfactory, is somewhat slower.

We have encountered instances in which errors have been made in assessing the efficiency of pasteurization by the phosphatase test because a "blank" test was omitted. We usually carry out such a test, in which a test-tube containing buffer substrate and chloroform but no milk is incubated with the principal tubes, with each batch of samples. Such a blank should give not more than 0·5 Lovibond blue unit. The substrate, disodium phenyl phosphate, is stable for at least 2 years when not in solution, but solutions of buffer substrate should be used within about 24 hr., unless shown later by test to be satisfactory.

Temperature of incubation

In the longer test (test B) which we almost invariably use, the incubation temperature recommended is 37–38° C. The latter temperature should not be exceeded. Temperatures between 34 and 38° have been found by experiment to give equally satisfactory results.

Time of incubation

For test B, the time of incubation originally adopted was 24 hr. In most laboratories this is a convenient time, allowing a steady routine during the day, in which samples are first prepared for incubation, and subsequently the

incubated samples from the previous day are removed from the water bath, precipitated, filtered and then the "blue units" measured.

In some cases it may be more convenient to terminate the incubation before 24 hr. have expired, and we have found in practice that a shortening of the incubation period to 20 hr. gives, with properly pasteurized and slightly under-pasteurized samples, very little difference from the usual 24 hr. period. For maximum sensitivity we do not consider it desirable to shorten the incubation period below 20 hr.

For official purposes it is better to keep to the original time of 24 hr., though a margin of ± 1 hr. is not significant.

The condition of raw milk and its effect on the heat stability of milk phosphatase

Quantities of fresh mixed herd milk were kept at 15 and 28° C. At 6 hr. intervals samples were removed from the bulk, pasteurized at $145 \pm 0{\cdot}1$° F. for 10, 20, and 30 min., and the following tests then performed.

On the raw milk:

Phosphatase by test "A" with controls. Methylene blue(8) at 37° C. (official method). *p*H using glass electrode.

Keeping quality, by boiling and smell.

Titratable acidity, with a few samples only.

Plate count and "coliform" test at 22° C. (using 1% milk agar, and bile salt lactose broth tubes incubated 5 days).

On the heated milk:

Plate count and "coliform" test at 22° C.

Phosphatase by test "B", in duplicate and with buffer alone, with the usual control tests.

From the results obtained it may be stated that there was no change in the heat stability of the enzyme (except perhaps for a slight reduction a few hours after milking) until there was a noticeable change in the condition of the raw milk which, of course, occurs after a few hours with milk produced under very dirty conditions, and after several days or more with very clean milk.

There was very close agreement between the results of the phosphatase tests on all the heated samples, until the milk, immediately prior to pasteurization, had a plate count of from 100 to 1000 million, a *p*H of 6·5 or less, a titratable acidity of 0·18 or more (as lactic acid), a methylene blue reduction time of under 1 hr. and a noticeable taint (bacterial).

The *p*H seems to be the influencing factor, for although milk may be tainted as a result of bacterial growth, until the natural change in *p*H falls outside the range 6·5 to 6·9 there is apparently no significant change in the heat stability of the enzyme.

Organisms producing substances which reduce Folin and Ciocalteu's reagent

While carrying out control[1] phosphatase tests on samples of milk of high bacterial content (about 500 million per ml.) we have found that many types of bacteria produce a substance (or substances) in milk which reduces the reagent of Folin and Ciocalteu (see also Jacobsen(13) and Burgwald & Gilbertson(14)). Such a substance is formed in milk by many proteolytic bacteria which produce a neutral or alkaline reaction in litmus milk, such as some of the green fluorescent organisms common in water and the spore-forming soil organisms, *B. cereus* and *B. megatherium*, etc.

Fortunately, as one would expect, these organisms do not produce sufficient reducing substances to interfere with the test until there are many millions present per ml. of milk (usually sufficient numbers to give a definite taint to the milk). Thus, if samples of milk are tested on the day of ~~production~~ pasteurization, or failing this, kept below 45° F. over a reasonable period, there is very little chance of such organisms giving trouble, i.e. being responsible for control values greater than 1·5 Lovibond blue units. We make a practice of discarding samples which give control values greater than 1·5 blue units, but they very rarely occur.

The following table shows that reducing substances are produced by *B. cereus* in sterile milk.

Table VI. *Control tests (unincubated), using ½ ml. (as in the phosphatase test) of the milk culture*

	Lovibond blue units	
Milk culture	Growth at 27° C.	Growth at 37° C.
Beginning	1·5	1·4
After 24 hr.	2·2	5·2 alk. clot
After 48 hr.	5·5	15·0

Many organisms produce the equivalent of over 0·4 mg. of phenol per 0·5 ml. of milk in cultures incubated for over a week at 37° C., e.g. *B. cereus* and *B. subtilis*.

The effect of bacteria capable of hydrolysing the substrate

The presence of bacteria capable of utilizing inorganic phosphates is of course to be expected(9,10,11). Many are capable of hydrolysing disodium phenyl phosphate. In the performance of the phosphatase test, however, if the substrate is correctly prepared, the chloroform prevents the growth of bacteria and in most cases destroys them, even when many millions of living cells are added with the milk.

However, only those bacterial types are of interest which can grow rapidly

[1] The milk is not incubated with the substrate but is added to the substrate plus Folin's reagent, and the test completed immediately (see H. D. Kay & W. R. Graham, *J. Dairy Res.* 1935, 6, 191).

in milk at normal temperatures without the production of substances reducing Folin's reagent, i.e. not giving rise to high control values, and particularly those which accompany rapid development with a slow change in the noticeable condition of the milk.

The most common organisms isolated from commercial pasteurized milk, have been non-heat resistant (destroyed in 10 min. at 145° F.) coliforms[1] probably derived from contaminated dairy plant. Both *Bact. coli* and *Bact. aerogenes* strains have been shown by us to hydrolyse the substrate, but the aerogenes strains have been most active.

In the absence of chloroform, the addition of 500 million cells (in $\frac{1}{2}$ ml. of milk) to the substrate has given rise to over 30 blue units, chiefly owing to the growth of the organisms. In the presence of chloroform the hydrolysis will be caused only, or chiefly, by the dead cells and seldom exceeds six blue units (test "B").

The "enzyme" is not destroyed by pasteurization and its presence may easily be verified by heating the milk or culture to 80° C. Five minutes in boiling water are, however, sufficient for its destruction in milk cultures at *p*H approximately 6·4.

During the past 5 years, a large number of naturally infected and experimentally inoculated milks have been tested under different conditions. On no occasion has bulk *raw milk* when pasteurized at 145 ± 0·1° F. for 30 min. given over 2·3 blue units when tested, unless the raw milk has had a marked taint (bacterial) and/or has clotted on boiling. Many samples gave values under 2·3 units, although they had plate counts (1% milk agar at 25–28° C. for 3 or more days) ranging from 600 million to 10,000 million per ml. just prior to pasteurization.

With *pasteurized milk*, naturally and experimentally inoculated, none of the organisms tested has caused an increase in the blue colour exceeding 2·3 units (controls being normal), except when the milk has been tainted (tested by smell before and after boiling), and has had a plate count of over 400 million per ml.

Although, on the basis of these findings there is little chance of difficulty in practice, some knowledge of the samples is necessary before testing for phosphatase (6). On no account should the test be made on tainted samples. All samples, whether kept under refrigeration or not, should be examined for taint by boiling 5 ml. in a test-tube for 2 or 3 min. It is most important, also, to use water saturated with chloroform when making the buffer substrate in order to be certain of preventing the growth of organisms capable of breaking down the substrate.

Table VII shows that, in the absence of bacteria capable of rapid hydrolysis of phenyl phosphate, large numbers of bacteria will not necessarily affect the result of the test.

[1] Short Gram-negative rods, producing acid and gas in MacConkey's bile salt lactose broth.

Table VII. *Pasteurized milk with* 0·2% *raw milk* (1) *and* 1·0% *raw milk* (2)

	Lovibond blue units	
4. ii. 37	(1)	(2)
A. Test "B" with chloroform	2·5	7·7
	2·5	7·7
B. Test "B" without chloroform	2·5	7·7
	2·5	7·6
Plate count of B before incubation	1,000,000 per ml. Coliforms present in 1/10,000 dilutions	
Plate count of B after incubation	220,000,000 per ml. Coliforms present in 1/1,000,000 dilution	
Plate count of A after incubation	Nil (on 1/10 ml. plate)	

MOULDS IN THE BUFFER SUBSTRATE

Buffer substrate made from tablets is usually more difficult to keep free from contamination than that made from the powder, and even when kept in the refrigerator moulds are a source of trouble. When tablets are used it has been found advisable always to use fresh solutions.

THE USE OF MERCURIC CHLORIDE FOR PRESERVING MILK SAMPLES

Having in mind that many samples of pasteurized milk will not remain sweet for 24 hr. at 70° F., and that the dispatch of samples in ice-boxes is expensive and sometimes impracticable, it was thought that preservatives which would prolong the life of milk without affecting the enzyme might be used. Since this work was completed other investigators (7) have reported on the use of preservatives and have arrived at similar conclusions.

From the results obtained so far, it seems that the addition of mercuric chloride, one part in 10,000 parts of milk, may be used without any significant effect on the milk phosphatase. The procedure was to add 1 ml. of a 1% solution of mercuric chloride to each 100 ml. of milk, e.g. 5 ml. to a pint (Imperial) of milk. Concentrations of 1 part in 5000 parts of milk, or higher concentrations, had some destructive or retarding action on the phosphatase. One part in 10,000, however, had no effect on the phosphatase test but increased the keeping quality of the milk from 1 to 8 days at 68° F.

SUMMARY

1. By the use of sodium hexametaphosphate it is possible to eliminate the final filtration in the phosphatase test, and thus appreciably to shorten the time required for the test.

2. It is necessary to avoid direct sunlight during that part of the test in which Folin's reagent is in use. Other minor precautions are mentioned in the paper.

3. The *p*H of the milk samples tested was found to vary naturally before pasteurization from *p*H 6·5 to 6·9 without affecting the validity of the test.

4. The test is also unaffected by the presence in milk of bacteria which produce reducing substances or which hydrolyse the substrate (sodium phenyl phosphate) unless the organisms are present in very large numbers.

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242. A RAPID PHOSPHATASE TEST

(A) ADAPTATION OF SCHARER'S MODIFICATION TO PASTEURIZING CONDITIONS IN GREAT BRITAIN; (B) A STUDY OF FACTORS INFLUENCING THE RELIABILITY OF THE TEST

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(*A*) Adaptation of Scharer's modification to pasteurizing conditions in Great Britain

Although Kay & Graham's phosphatase test (1) has proved its great practical value, it has been felt that a more rapid method of determining the phosphatase in pasteurized milk would be of definite advantage, in particular to industrial laboratories, provided that the degree of sensitivity of the original method could be maintained. It is claimed (2,3) that two modifications, introduced by Scharer, fulfil this requirement under American conditions of pasteurization (holding at 143° F. for 30 min.) and we have considered their possible adaptation to the more severe pasteurizing conditions in Great Britain (holding at 145° F. for 30 min.).

In order to detect the smaller amounts of residual phosphatase which remain active after heating milk for 30 min. at temperatures *above* 143° F., it is necessary to extend the period of incubation in the tests. With Scharer's "one hour laboratory test" an incubation time of the order of 3 hr. was required, an inconvenient time from the practical point of view,[1] whereas with the so-called "10 min. field test" half an hour's incubation was found adequate and incubation for 1 hr. led to a notable increase in precision. This latter experience corresponds to that of certain American workers (5,6) who observed that incubation for 20–30 instead of 10 min. improved the sensitivity of the field test. Incubation for 40 min. at *47*° C. appeared to give results equivalent to 1 hr. incubation at 37° C., but as most laboratories are equipped with 37° C.-incubators, we preferred to adopt this temperature.

A number of minor modifications were introduced during the development of the test which, it is felt, is now as searching as the 24 hr. test of Kay & Graham.

Method

Reagents.

(1) *Buffer:* 0·5 *N* Na carbonate (dissolve 2·65 g. of anhydrous Na_2CO_3 (C.P.) in 100 ml. of distilled water).

Stable for at least 2 weeks, if kept stoppered. It was suggested early in our investigations that a simple carbonate buffer could be used with advantage in place of the borate buffer recommended by Scharer.

[1] Recently, Jepsen & Madelung (4) have shown that a highly sensitive test is obtained by increasing the time of incubation to 20 hr. and by raising, at the same time, the colour standard, but this procedure robs the test of its main advantage, speed.

(2) *Buffer substrate:* dissolve 0·11 g. disodium phenyl phosphate in 80–90 ml. of distilled water, add 3 ml. of buffer and make up to 100 ml.

This solution should be used within a few hours of preparation although, on occasion, it has been observed to remain stable for several days when kept in a refrigerator. Disodium phenyl phosphate sufficiently free from phenol to require no further laboratory purification is now commercially obtainable from British Drug Houses, Ltd. It should be kept in a refrigerator. Fresh supplies should be tested before acceptance. Chloroform should not be used in the preparation of the buffer substrate solution, as it interferes with the extraction of the indophenol colour into butyl alcohol. Excess chloroform greatly retards this process.

(3) *Gibbs' phenol reagent:* dissolve $23 \pm 0{\cdot}5$ mg. of 2:6-dibromo quinone chloroimide (B.Q.C.) in 5 ml. of absolute alcohol.

Keep in a good quality *brown* bottle. The reagent is stable for a week at room temperature and for a month when kept at a temperature below 45° F. According to Scharer[1] the solution should be discarded as soon as it shows signs of becoming brown or reddish brown. Care should be taken to use only pure, canary yellow B.Q.C. powder in preparing the solution. Solutions made up from brownish and discoloured powder produce "off colours" in the test which cannot be compared with the colour standards.

(4) *Normal butyl alcohol:* neutralize by adding NaOH, using thymol blue or other convenient indicator.

500 ml. of a good quality alcohol obtained from B.D.H. required usually 0·3 ml. of *N*/2 NaOH. Although "industrial" butyl alcohol has been found satisfactory, we recommend the use of a better grade.

Folley & Kay(7) determined the optimum concentration for hydrolysis of mammary gland phosphatase at *p*H 10 to be 0·009 m. disodium phenyl phosphate. Using heated milk containing residual phosphatase, we found only a very small progressive increase in the rate of hydrolysis with substrate concentrations above 0·004 m. We therefore decided to adhere to the concentration of 0·005 m. used in all previous tests for milk phosphatase.

The mixture of milk and buffer substrate solution obtained when using Scharer's technique has a *p*H of approx. 9 at 37° C. Since Folley & Kay showed that optimum hydrolysis of mammary gland phosphatase takes place at *p*H 10, we have altered the composition of the buffer substrate solution so as to give a *p*H of this order after addition of the milk. The buffer substrate solution, recommended above, has a *p*H of 10·7–10·8 at 18° C. Small variations in the amount of buffer added are of little account, addition of 2·5 or 3·5 instead of 3·0 ml. changing the *p*H, for instance, from 10·77 to only 10·73 and 10·83 respectively. Addition of the milk to the buffer substrate reduces the *p*H to about 10·2 which corresponds to a *p*H of approximately 10 at 37° C. Optimum hydrolysis was actually obtained in substrate solutions containing only 2 ml. of carbonate buffer, but greater consistency was achieved by raising the amount of buffer to 3 ml., probably on account of the increased buffering power of the stronger solution.

[1] Private communication.

Apparatus

Pipettes: a few 5 ml. pipettes and a number of 1 and 10 ml. graduated pipettes.

Waterbath: maintained at 37–38° C.

Test-tubes: length 150 mm., internal diameter 13·5 ± 0·5 mm.

It is important to use test-tubes of the above dimensions. Experience has shown that the use of smaller test-tubes leads to markedly reduced extraction of the indophenol colour developed in the test, whilst inconsistencies are encountered when using tubes of larger size.

Rubber stoppers: to fit test-tubes. They should be kept in water and boiled before use.

Glass dropper: for B.Q.C. solution.

This should be tested to deliver 50 ± 2 drops of B.Q.C. solution per ml. Use in vertical position.

Lovibond tintometer: if accurate colour readings are required.

Technique

(1) Transfer 5 ml. of freshly prepared buffer-substrate solution to a test-tube and add 0·5 ml. of the well-mixed milk.

(2) Stopper, shake 8–10 times and incubate for 1 hr. at 37–38° C.

Incubate, at the same time, a "blank", i.e. 5 ml. of buffer-substrate solution to which no milk has been added.

(3) After 1 hr., take the tubes from the bath, remove stoppers and start at once to add 5 drops of B.Q.C. solution. Add to one or two tubes at a time and mix immediately, using about ten circular motions. (If the contents of the test-tubes are allowed to cool below 30° C. before addition of the B.Q.C. solution, variations in the strength and shade of indophenol colour will occur. To avoid such variations it is also essential to use exactly 5 drops of B.Q.C. solution.) After all the tubes have been thus treated, replace stoppers and shake about 10 times. Use only 1 drop of B.Q.C. solution for the "blank" test.

(4) Remove stoppers, add 2 ml. of butylalcohol and replace stoppers.

(5) Cool the tubes to 15–20° C. Commence to extract the indophenol colour 5–10 min. after the addition of the B.Q.C. solution. Working with pairs, invert the tubes slowly 8 times (4 complete turns). Replace the tubes in a rack after each inversion and do not invert again until the two layers have clearly separated.

The tubes should be tilted slowly until the liquid just reaches the opposite end and then brought to the vertical position a little more quickly in such a manner that about ½ min. is required for the two layers to separate clearly.[1]

Experience has shown that unsatisfactory results are obtained when the test solutions are at temperatures above 28 or below 10° C., and that the quickest and clearest separation is achieved by cooling the tubes to 15–20° C.

[1] With a little practice it is quite easy to obtain maximum extraction of the indophenol colour and to avoid delay in the separation of the layers caused by too rapid inversion of the tubes.

The process of extraction takes about 10 min. whether working with two or twelve tubes; in the latter case there is no waiting between each inversion. The colour extraction should be completed within 20 min. after removal of the tubes from the water bath.

(6) Read the colour developed in the butylalcohol layer. (See below.)

"*Control tests.*" In addition to "blank" tests which should be carried out with each batch of tests, it is necessary to perform control tests in all cases in which it is suggested that the colour development in the test proper might be due to causes other than milk phosphatase (such as contamination by phenolic disinfectants).

Add 0·5 ml. of the milk to 5 ml. of a solution containing 3 ml. of the carbonate buffer, but no substrate, in 100 ml. of water, incubate for 1 hr. at 37–38° C. or just warm to this temperature and then proceed as usual.

Methods of colour reading.

(*a*) By means of the Lovibond tintometer. For accurate readings of the indophenol colour the Lovibond tintometer may be used. The butyl alcohol layer is syphoned off and transferred to a $\frac{1}{2}$ in. square cell (as used in the Hellige comparator), but as 2 ml. are insufficient to give the depth of liquid required to cover the opening of the tintometer, it is necessary to perform the phosphatase test in duplicate and to combine the butyl alcohol layers. As the size of test-tubes affects the colour extraction, more consistent results are obtained in this manner than by performing the test in larger test-tubes using double quantities. The indophenol colour is matched by the use of blue, yellow and "neutral" glasses.

(*b*) By means of inorganic colour standards. For routine testing the quicker method of comparing the colour of the butyl alcohol layer with that of one or more colour standards is more satisfactory.

Following Scharer's recommendation(3) three stock solutions are made up for the preparation of the colour standards:

Colour	Salt	g.
Blue	$CuSO_4.5H_2O$	6·243
Yellow	$FeCl_3.6H_2O$	4·505
Red	$CoCl_2.6H_2O$	5·959

Dissolve in, and make up to 100 ml. with, 1% HCl solution (*w*/*v*).

The actual colour standards are prepared by mixing these solutions and water in the following proportions:

ml.	Blue	Yellow	Red	H_2O
I	4·6	3·0	0·9	1·5
II	5·5	2·7	0·8	1·0

These correspond within ±0·1 unit to:

Lovibond units	Blue	Yellow	Neutral
I	2·0	2·4	0·4
II	2·5	2·5	0·6

These standards were evolved by analysing the results of phosphatase tests on samples of mixed milk accurately heated at 145° F. for 20 and 30 min.

It was found that with milk mixed in quantities of 100 gallons or over the average colour readings were as follows, with a minimum of 3·0 blue units for milk heated for 20 min., and a maximum of 1·6 blue units for milk heated for 30 min.

Lovibond units	Blue	Yellow	Neutral
Pasteurized at 145° F. for 20 min.	**3·75**	3·25	0·95
Pasteurized at 145° F. for 30 min.	**1·25**	1·75	0·25

The colour of standard I is therefore well above that given by properly pasteurized milk, whilst standard II is sufficiently low to help in detecting a reduction in heating time of less than 10 min. (or, correspondingly, a drop in temperature of less than $1\frac{1}{2}$° F. or contamination by small quantities of raw milk).

As will be discussed later, colour readings of less than 3·0 blue units after heating at 145° F. for 20 min. and of more than 2·0 blue units after pasteurization for 30 min. are occasionally encountered when dealing with milks bulked in quantities of less than 100 gallons. For practical purposes it is, therefore, recommended to leave a greater margin in favour of the distributer and not to condemn a sample as under-pasteurized unless it gives a colour reading above standard II, although a sample giving a reading between standards I and II may, in general, be regarded as "doubtful".

The colour standards should be kept in the dark and checked at frequent intervals. They are stable for at least one month.

For comparison of the colour extracted in the test with the standards, it is recommended to examine the test-tubes in reflected light, holding them close to a white background, and to interchange their positions. No attempt should be made to match the colours, but rather to determine whether the test solutions contain more or less of the blue component than the standards.

The preparation of permanent standards made from Lovibond glasses is under consideration.

Interpretation of results

A. Five minutes after the addition of B.Q.C. solution:

(1) A buff brown or grey brown colour is given by milk heated for 20 min. or more at 145° F. or for 30 min. at a higher temperature than 143° F.;

(2) A *blue-grey* colour is given by milk heated for 15 min. at 145° F. or for 30 min. at 143° F. or by properly pasteurized milk containing 0·5 % of raw milk;

(3) A deep blue colour is given by raw milk immediately on adding the B.Q.C. solution.

B. After extraction with butylalcohol:

(1) A greenish yellow or yellowish green colour is given by milk heated for 30 min. at 145° F. (in general, below 2·0 Lovibond blue units or a weaker colour than standard I);[1]

[1] An absence of greenish colour indicates that the milk has had a more severe heat treatment.

(2) A green or blue-green colour is given by milk heated at 145° F. for 20 min., or heated at 143·5° F. for 30 min., or heated at 144° F. for 25 min., or by correctly pasteurized milk containing 0·2% of raw milk (usually 3–5 Lovibond blue units or a stronger colour than standard II). In our experience slightly more blue colour is developed by milk heated at 143·5° F. for 30 min. than by milk heated at 145° F. for 20 min.

"*Blank*"-*tests*. No greenish or bluish colour should develop either before or after extraction. In case of colour development test buffer and substrate separately.

"*Control*" *tests*. Normal milk should give a colourless or yellowish butyl alcohol layer. A green or blue colour indicates the presence of phenol. With milk in an advanced stage of decomposition, phenol may be produced by bacteria.

A quick test for raw milk.

A quick test to distinguish raw from heated milk can be obtained by adding 5 drops of B.Q.C. solution to a mixture of 5 ml. of buffer-substrate and 0·5 ml. of milk at room temperature (15–20° C.). In the presence of raw milk a bluish colour develops in less than 3 minutes.

Results

The results recorded below were obtained by accurate laboratory pasteurization of 10 ml. samples of well-mixed milk in a thermostat at 145 ± 0·2° F. In general, each batch consisted of sixteen tubes of which two contained control thermometers. The tubes were totally immersed and mechanically rotated around a horizontal axis. They reached pasteurizing temperature within 1–2 min. and were immediately cooled in ice water after removal from the bath.

Bulk milk and, for special purposes, milk from single cows was examined. The milk was pasteurized within 24 hr. of milking, precautions being taken to exclude "tainted" samples.

Comparison with the Kay & Graham 24 hr. test.

Table I contains representative results obtained from tests on the same milk by the rapid method and the 24 hr. Kay & Graham test (average readings of duplicates).

Table I

		Lovibond blue units	
Milk	Heat treatment	Rapid test	Kay & Graham test
1	30 min. 145° F.	1·2	1·5
1	20 min. 145° F.	3·1	2·4
1	30 min. 144° F.	2·1	1·9
1	25 min. 144° F.	3·3	2·7
1	15 min. 144° F.	14·0	8·8
5	30 min. 145° F.	1·3	1·4
5	20 min. 145° F.	4·5	3·1

These figures show that the same reduction in the time of heating increases the colour strength in the rapid test by about twice the amount of additional colour produced in the Kay & Graham test. It follows that an error in colour reading affects the rapid test to a lesser degree than the Kay & Graham test.

Accuracy of laboratory pasteurization.

In Table II, a few results are given showing the accuracy obtained by repeat pasteurization of samples of the same milk.

Table II

Milk	Heat treatment at 145° F. min.	Lovibond units Blue	Yellow	Neutral	Particulars
4	30	1·0	1·6	0·1	
4	30	0·9	1·7	0·1	Pasteurized 1 hr. later
4	20	3·9	3·1	1·0	
4	20	4·1	3·5	1·0	Pasteurized 1 hr. later

The effect of storage. It was found that properly pasteurized as well as under-pasteurized milk gave results which were practically identical with those obtained directly after heat treatment, when milk was kept for periods of 2–8 days at a temperature of 35–50° F., provided the milk did not show a noticeable bacterial taint at the time of retesting.

Tests on mixed milk.

(*a*) *Bulked in quantities of* 100 *gallons and over.* Forty samples were tested after pasteurization at 145° F. for 20 and 30 min. Representative results are given in Table III.

Table III

Sample no.	Gallons	Phosphatase in Lovibond units: Raw milk*	Milk heated at 145° F. For 30 min. Blue	Yellow	Neutral	For 20 min. Blue	Yellow	Neutral
3	100	4·3	0·8	1·4	0	3·0	2·9	0·8
56	100	5·1	1·2	1·6	0·3	3·9	3·3	1·0
52	150	—	1·5	1·8	0·3	4·0	3·5	1·0
50	160	5·8	1·3	1·6	0·2	4·0	3·3	1·0
42	150	—	1·1	1·6	0·2	4·3	3·8	1·0
45	200	5·1	1·6	2·1	0·3	4·1	3·5	1·0
47	200	4·5	1·3	1·8	0·3	3·7	3·1	1·0
43	230	—	1·3	1·8	0·3	4·1	3·6	1·0
53	250	5·1	1·3	1·6	0·3	3·5	3·0	0·9
49	1000	5·1	1·4	1·7	0·3	3·8	3·1	1·0

* Determined by a slight modification of Kay & Graham's test "A". Milk is diluted 1 : 5 with water and an incubation time of ½ hr. at 37° C. is used.

All forty samples gave values of less than 2·0 Lovibond blue units after pasteurization for 30 min. Thirty-nine out of forty samples gave values above 3·0 blue units after heating for 20 min., the remaining sample giving between 2·5 and 3·0 blue units. It was noted that this sample contained the milk of one herd only. The anomalous result may, therefore, be due to adulteration

(addition of water or removal of cream) which is more likely to occur with milk from a single herd.

(*b*) *Bulked in quantities of less than* 100 *gallons.* 100 samples were tested after being pasteurized in the same manner.

The following results were obtained:

	Lovibond blue units	Number of samples
(*a*) Pasteurized at 145° F. for 30 min.	<2·0	93
	2·0–2·5	5
	>2·5	2*
(*b*) Pasteurized at 145° F. for 20 min.	>3·0	85
	2·5–3·0	13
	<2·5	2†

* From bulks of 30 and 40 gallons respectively. † From bulks of 11 and 75 gallons respectively.

The results show that deviations from the normal amounts of colour developed in the test are encountered when testing samples made up from the milk of a comparatively small number of animals. Although in actual practice pasteurization of such samples will rarely occur in this country, it was felt that these deviations ought, nevertheless, to be taken into account in considering the colour standards to be used in the interpretation of the test. It was, therefore, recommended that two standards should be laid down, corresponding to 2·0 and 2·5 Lovibond blue units, and that all samples giving more than 2·5 blue units should be classified as "under-pasteurized" and that all samples giving between 2·0 and 2·5 units should be classified as "doubtful". In this manner only two instead of seven out of 100 samples which had been properly pasteurized fail to pass the phosphatase test, although the remaining five are still, wrongly, regarded as doubtful. This proposal appears to be fairest to the operator of the pasteurizing plant, although by raising the standard from 2·0 to 2·5 blue units, some of the samples heated for only 20 min. at 145° F. are classified as doubtful instead of as definitely under-pasteurized.

The deviations would appear to be due to the fact that the behaviour of the phosphatase present in any one cow's milk makes itself more and more felt as the number of animals whose milk is used for mixing decreases. It is known that the amount of enzyme in raw milk varies considerably with the stage of lactation, and between individual animals. Little is known, however, regarding the amount of enzyme remaining active after heat treatment in relation to the stage of lactation or to the phosphatase concentration in raw milk and regarding other factors such as possible changes in the rate of destruction of the enzyme due to udder infections. The results given below are, therefore, of interest.

(*B*) A STUDY OF FACTORS INFLUENCING THE RELIABILITY OF THE TEST

Effect of stage of lactation

Folley & Kay(8) have shown that in *raw* milk the amount of phosphatase per unit volume reaches a minimum a few days after parturition, after which it increases as lactation progresses. At the same time, their data show that wide

differences in enzyme concentration exist between the milk of individual cows at any particular stage of lactation.

The representative results given in Table IV show that there does not appear to be any marked correlation between residual phosphatase and either the stage of lactation or the concentration of the enzyme in the raw milk, except, perhaps, during the first few weeks after parturition, when low phosphatase readings are usually found both before and after heat treatment.

Table IV

				Phosphatase in blue units		
					Milk heated at 145° F. for	
Cow	Stage of lactation	Daily yield in lb.	*p*H	Raw milk*	30 min.	20 min.
RR2	Colostrum 12 hr. after parturition	—	6·28	2·8	2·5	3·0
19	1 week	36	—	3·8	1·5	2·5
1	2 weeks	28	—	2·0	1·5	2·0
7	3 weeks	44	—	1·8	1·0	1·5
38	1–2 months	29	—	—	1·0	2·8
32	,,	34	—	—	1·0	1·5
8	,,	42	—	10·6	2·7†	6·5
6	,,	35	—	—	1·0	2·8
5	2–4 months	36	—	—	1·5	4·0
20	,,	22	—	—	2·0	4·5
15	,,	39	—	—	1·5	3·0
26	,,	13	—	—	1·5	5·0
12‡	4–8 months	4	7·14	7·2	0·5	1·0
24	,,	15	—	7·6	1·5	4·0
18	,,	5	6·58	—	1·5	4·0
23	,,	30	—	—	1·5	4·5
22	,,	12	6·70	—	1·7	4·5
35	,,	12	6·80	14·4	2·2†	4·0
21‡	,,	21	—	—	1·0	1·5
2	8–10 months	16	7·24	5·0	1·1	4·0
10	,,	12	—	17·2	1·0	3·0
9‡	16 months	7	—	4·3	1·5	2·5
31	17 months	14	—	—	1·0	3·0

* By modified Kay & Graham test "A" (see Table III). † Control negative.

‡ Milked once a day. It is remarkable that these cases show particularly low values for residual phosphatase after 20 min. heating. No evidence for clinical mastitis was found. The high *p*H value and low yield in case 12 strongly indicate disturbed secretion.

Similar results were obtained by examination of mixed milk from cows grouped according to the stage of lactation (Table V). In this series milk from the Institute's herd was used and care was taken to include only the milk from healthy animals.

In another series of this kind (Table VI), carried out 2 months earlier (and repeated within 48 hr. with almost identical results), the milk from the group of animals less than 60 days in lactation did not give low values for residual phosphatase, although the phosphatase concentration in the raw milk was quite low. In this series the milk from cows suffering from slight udder infections had not been excluded, but it is doubtful whether this fact can serve as an explanation for the discrepancy from the results obtained later. The observation certainly emphasizes the great caution needed in interpreting results obtained by the examination of milk from small groups of animals.

Table V

No. of cows	Lactation group days in milk	*p*H	Phosphatase in blue units. Milk heated at 145° F. for 30 min.	Phosphatase in blue units. Milk heated at 145° F. for 20 min.
5	Under 60	—	0·9	2·0
7	60–120	6·70	0·8	4·1
7	120–180	6·76	1·0	3·9
9	180–240	6·75	0·9	2·7
5	240–300	6·76	1·2	4·6
4	300–360	—	1·3	4·0
2	Over 360	—	1·5	3·4
39	Bulk sample	6·71	1·1	4·1

Table VI

No. of cows	Lactation group days in milk	*p*H	Chloride %	Phosphatase in blue units. Raw milk*	Phosphatase in blue units. Milk heated at 145° F. for 30 min.	Phosphatase in blue units. Milk heated at 145° F. for 20 min.
6†	10–60	6·64	0·076	3·5	1·5	4·0
6	150–250	6·70	0·096	10·0	1·8	4·5
6	Above 280	6·90	0·127	14·5	1·5	3·5
2	Above 450	6·76	0·104	12·5	1·5	3·0

* By modified Kay & Graham test "A" (see Table III).

† Two suffering from slight udder infections; not *Str. agalactiae*.

Although inconclusive, the experiments given in Tables IV–VI indicate that there is some danger that bulk samples containing a very high percentage of milk from cows in the early stages of lactation may give negative phosphatase tests after under-pasteurization such as heating for only 20 min. at 145° F.

An interesting observation, for which we have as yet no explanation, is the lack of proportionality between the residual enzyme activity after 20 and that after 30 min. heating at 145° F. This cannot be due to experimental errors in the heating or testing of milk, since close agreement was obtained whenever experiments with any particular sample were duplicated (see Table II).

Effect of mastitis

In view of the prevalence of mastitis and the often voiced but never confirmed suspicion that abnormal phosphatase tests result when testing "mastitis milk", we have examined the milk of a few cows suffering from non-clinical udder infections.

In May 1939 samples were taken from thirteen cows, twelve of which had been shown, in February, to suffer from udder infections.[1] Bacteriological and phosphatase tests were carried out on these samples.

For the bacteriological tests, samples were taken from the foremilk after withdrawal of the first 5 or 10 ml. and the milk was plated within a few hours on Edwards' medium (9), using the centrifuged deposit method (10). Large numbers of colonies showing "weak β" haemolysis were regarded as indicative of infection with *Str. agalactiae*. Other organisms were subjected to further bacterial and biochemical tests (11, 12).

[1] We are indebted to Dr J. G. Davis for this information.

For the phosphatase tests samples were taken after the cows had been milked out. The milk was pasteurized in the laboratory about 10 hr. later. The results are given in Table VII.

Table VII

No.	Months in milk	Daily yield in lb.	Diagnosis Feb.	Diagnosis May	Phosphatase in blue units: Milk heated at 145° F. for 30 min. Kay & Graham 24 hr. test	30 min. rapid test	20 min. rapid test	Raw milk*
3	7	19	*Str. agalactiae*	*Str. agalactiae*†	1·9	1·6	4·0	9·1
8	5	22	,,	,,†	2·1	1·6	4·0	11·5
6	4	17	,,	,,†	2·8	4·1‡	6·5	14·0
15	5	25	,,	,,†	2·8	3·4‡	5·0	9·5
9	2	31	Not ,,	Mixed	—	2·0	2·6	—
11	9	20	Not ,,	,,	—	1·6	3·0	—
7	1	35	,,	,,	—	1·2	3·5	—
4	5	18	,,	?	—	2·0	4·0	—
5	5½	28	,,	?	—	2·0	4·0	—
10	5	16	Not ,,	?	—	1·1	2·4	—
14	1 week	29	Not ,,	0	—	1·2	2·4	—
13	4	29	?	?	—	2·0	2·8	—
12	5½	40	0	0	—	1·5	3·0	—
16	Above samples mixed in equal quantities					1·8	3·7	—
17			,,	proportion to yield		1·4	4·0	—

* By modified Kay & Graham test "A" (see Table III).

† Over 300 colonies per ml. of milk, showing "weak β" haemolysis. Had given positive tests in January 1938, i.e. during previous lactation.

‡ Control negative.

Thus four cows were severely infected with *Str. agalactiae*, and three had a less severe mixed infection, whilst the remaining animals were doubtful or negative cases. Only two of the animals (nos. 6 and 15), however, were found to secrete milk which gave a high phosphatase value after correct pasteurization. Accordingly, nothing abnormal was found when testing the mixed milk of the thirteen animals (nos. 16 and 17), which may be regarded as representative of milk from a herd containing over 50% of infected animals. However, as only four of the animals could be regarded as definite cases of *Str. agalactiae* infection, further experiments were carried out in order to ascertain the proportion of mastitis milk needed to cause a positive phosphatase test after proper pasteurization. For this purpose milk from the two cows giving abnormally high colour readings (nos. 6 and 15) were mixed in equal quantities and added in different proportions to bulk milk from animals free from udder infections. After heat treatment in the usual manner, phosphatase tests resulted as follows (Table VIII).

Even in this case more than 50% of "mastitis" milk was required to give a phosphatase test indicative of under-pasteurization after the milk had been correctly pasteurized, although 35–50% sufficed, with the rapid test, to produce colour readings leading to a classification of the samples as "doubtful".

One of the cows (no. 6) giving abnormally high colour readings after proper pasteurization reacted positively to the B.C.P. test on the hindquarters only. Separate samples of the milk of the hind- and forequarters were, therefore,

Table VIII

	Phosphatase in blue units. Milk heated at 145° F. for 30 min.	
	Kay & Graham 24 hr. test	Rapid test
Bulk milk, no mastitis	1·6	1·5
,, +25% mastitis milk	—	1·7
,, +35% ,,	—	2·1
,, +50% ,,	2·2	2·4
,, +60% ,,	—	2·8
Mastitis milk alone	2·8*	3·4

* Control = 1·5 blue units.

taken one week later to determine whether the high phosphatase value given by the mixed sample taken on the first occasion was due to mastitis or other unknown causes. The repeat samples were tested bacteriologically and pasteurized both separately and after mixing (Table IX).

Table IX

Date of sampling	Description of sample	No. of "weak β"* haemolytic colonies per ml. of milk	Titr. acidity	*p*H	Phosphatase in blue units. Raw milk†	Milk heated at 145° F. for 30 min.	Milk heated at 145° F. for 20 min.
23. v. 39 a.m.	Mixed quarters	Over 350‡	—	—	14	4·1	6·5
1. vi. 39 p.m.	Forequarters	Over 60	0·16	6·68	5·5	1·4	3·4
	Hindquarters	Over 6000	0·14	6·78	7·3	2·0	3·5
	Mixed quarters	—	—	6·70	6·6	1·7	3·6

* Edwards' medium. † By modified Kay & Graham test (see Table III). ‡ Foremilk.

The results show that the phosphatase figures fluctuate considerably, before as well as after heat treatment, neither the mixed milk nor that of the severely infected hindquarters giving abnormally high results at the second sampling. Owing to these fluctuations it cannot be assumed that the high value obtained in the first instance was due to mastitis, although the fact that the milk from the hindquarters still gave higher colour readings after correct pasteurization than that of the forequarters points in that direction. The variations in enzyme activity observed in this case provide a likely explanation for the fact that two of the four definite cases of mastitis (Table VII, nos. 3 and 8) gave normal phosphatase tests after correct pasteurization.

The effect of clinical mastitis was investigated in only one instance. A mixture of the milk of two cows showing clinical evidence of the disease was obtained. The milk was of brownish-yellow colour and contained thick clots. It had a *p*H of 6·86. The phosphatase activity of the raw milk was very high, corresponding to 22 blue units when tested by the modified Kay & Graham test "A". After pasteurization at 145° F. for 30 min. a colour reading of 3·0 blue units resulted, viz. a reading exceeding the permissible standard, but not very high considering the severity of the infection.

The limited number of experiments indicates that changes caused in the

milk by mastitis may increase both the phosphatase in raw milk and that remaining after heat treatment. Thus abnormally high colour readings may be recorded after correct pasteurization. Unless more than 50% of the cows contributing to a large bulk of milk are suffering from mastitis, this is unlikely to occur in practice.

Anomalies

As has been mentioned on p. 492, two out of 100 samples of milk bulked in quantities of less than 100 gallons showed abnormally high phosphatase activity after correct pasteurization. In both cases the sample contained the mixed milk of a single herd. Investigations of the milk of these herds, still in progress, indicate that factors other than those discussed above are likely to be the cause of the anomalies noted at the first sampling.

SUMMARY

By adapting Scharer's "10 min. field test" to British conditions of pasteurization, an accurate phosphatase test has been evolved requiring less than 1½ hr. for completion. The method is described in detail.

A small percentage of anomalies was detected when applying the new method to the examination of pasteurized samples bulked from small quantities of milk (less than 100 gallons). This led to a study of several factors thought to be the likely cause of such anomalous results.

No correlation was found between the phosphatase surviving heat treatment and the stage of lactation when milk from individual cows and from small groups of animals was tested, with the possible exception of the early stages of lactation when the enzyme concentration before and after heating was frequently found to be low.

It was observed that pasteurized milk from animals suffering from mastitis tends to give abnormally high results of the phosphatase test. The amount of residual phosphatase is subject to considerable fluctuation.

We wish to thank Prof. H. D. Kay and Dr A. T. R. Mattick for their helpful interest.

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243. FACTORS AFFECTING THE SOLUBILITY OF MILK POWDERS

IV. THE INFLUENCE OF SPEED AND DURATION OF STIRRING ON SOLUBILITY, WITH A DESCRIPTION OF A RAPID METHOD FOR SOLUBILITY DETERMINATIONS

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(With 7 Figures)

In the first paper of this series(1), the following method was adopted for the determination of the solubility of milk powders. Two g. of milk powder were mixed with 20 ml. of water, the solution stirred at 2000 r.p.m. for 30 min. at the desired temperature of reconstitution and then centrifuged for 15 min. at 3000 r.p.m. The amount of protein in the supernatant liquid, expressed as a percentage of the total protein, was taken as the percentage solubility. These conditions were naturally somewhat arbitrary, but it seemed reasonable to suppose that such treatment would successfully effect the solution of the soluble part of the powder and leave the insoluble residue unaffected.

In a series of routine determinations of the solubility of roller-dried separated milk samples in which this method was used, data were obtained which suggested that the speed at which a milk powder sample was stirred during the test had a far more marked effect on the value obtained for the solubility of the powder than had hitherto been realized, while the duration of stirring also materially affected the result. Since this is obviously a point of considerable importance in formulating standard methods for solubility determinations, a comprehensive set of tests was made on a number of samples. These tests confirmed the earlier observations, and it was considered that further investigation into the effect of speed and duration of stirring on the apparent solubility of dried milk powders was warranted.

During the progress of this part of the work experiments were also carried out to establish a method for estimating solubilities which would combine the reliability of the process hitherto used in this series of papers with very much greater rapidity. The first step towards this end was to compare for a number of samples the solubility values calculated on a protein basis with those obtained from the estimation of total solids, as total solids are more readily determined than nitrogen. The second step was to establish a rapid method for estimating solubilities using total solid estimations and replacing the 30 or 45 min. period of stirring by 1 min. shaking. The latter part of the present paper deals with a comparison of the results obtained by this rapid method for roller- and spray-

dried whole and separated milks with those obtained by the longer stirring process, and at the same time the rapid method is also compared with the sediment test commonly used in industry.

Part I. The effect of speed and duration of stirring on the apparent solubility

Experimental. To avoid any possible complications due to the presence of the fat phase in whole-milk powders, a roller-dried separated milk was used for this section of the work. A thoroughly representative batch of powder was obtained by mixing several samples all obtained from the same factory. This composite batch contained 34% protein, 8% ash and 4–6% moisture.

Three series of experiments were carried out, in each of which the speed of stirring was kept constant while the duration of stirring was varied. In the first series the stirring speed was 1100 r.p.m., in the second 2200 r.p.m. and in the third 4300 r.p.m., each value being maintained to within ± 100 r.p.m. The times for which the samples were stirred were 10, 20, 30, 45, 60, 100, 140 and 300 min., an additional allowance of 3 min. being made to permit the sample to reach the temperature of reconstitution. The temperature of reconstitution was kept constant, but determinations were made at both 20 and 50° C., as previous work(2) had shown that the solubility figures obtained at these temperatures are the minimum and maximum values between 20 and 100° C.

Apart from the variations in the duration of stirring the experimental technique was identical with that previously employed. Nitrogen was estimated by the micro-Kjeldahl method, while the total solids were determined in this section of the work according to the recommendation issued by the Milk Products Sub-Committee of the Society of Public Analysts for the analysis of condensed milk(3). The percentage solubility on a total solids basis was obtained by expressing the total solids in the supernatant liquid after centrifuging as a percentage of the total solids which would have been present if the powder had completely dissolved.

Results and discussion. Fig. 1 shows the effect of alterations in the speed and duration of stirring on the solubility of the proteins of the reconstituted milk powder. The outstanding feature of the results is the extraordinarily marked effect of the speed of stirring on the solubility of the powder. It will be seen that for each speed the protein solubility rises rapidly to a certain value beyond which there is little further increase. The value where this takes place is referred to here as the "equilibrium value". Thus with solutions reconstituted at 50° C. the equilibrium value is about 80% at 1100 r.p.m., 82% at 2200 r.p.m., and 89% at 4300 r.p.m. With solutions reconstituted at 20° C. the equivalent values are 53, 56 and 64% respectively. The time in which the equilibrium figure at 20° C. is reached varies with the speed of stirring; at 4300 r.p.m. it is reached in 30–35 min., at 2200 r.p.m. in 60 min., and at 1100 r.p.m. in between 100 and 150 min. This variation is reflected in differences in the shape of the

curves at the three speeds. With a speed of 4300 r.p.m. there is a definite break in the curve at the equilibrium point, with a speed of 2200 r.p.m. the break is difference is less definite, while with a speed of 1100 there is no perceptible break, and the difference is shown simply as a gradual change in the gradient of the curve. Once the equilibrium level has been attained, however, the subsequent increase in solubility is extremely slow, the curves rising along parallel lines at the rate of only about 1% per hour.

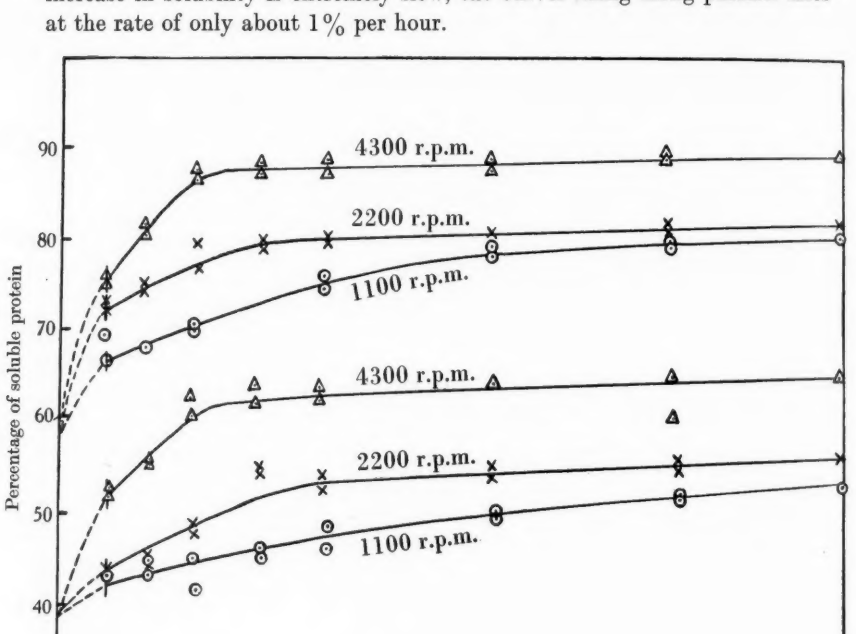

Fig. 1. Effect of duration and speed of stirring on the protein solubility; upper three curves at 50° C., lower three curves at 20° C.

In order to gauge more exactly the effect of speed of stirring on solubility a series of experiments was carried out in which the duration of stirring was kept constant at 45 min. while the stirring speed was varied between 1000 and 5300 r.p.m. The results of these experiments are shown in Fig. 2. It will be seen that the points fall roughly on a straight line, and that for each increase in stirring speed of 1000 r.p.m. the solubility increases by about 4%.[1] Owing to excessive frothing at high speeds of stirring it was not found practicable to use speeds over 5300 r.p.m.

[1] I.e. from, say, 72 to 76%. Throughout this paper percentage differences are expressed on this basis and not as percentage changes from the first value. The latter method would give a difference of $\left(\frac{76-72}{72}\right) \times 100$, or 5·5%.

An experiment was, however, carried out in which the milk powder was reconstituted without any mechanical stirring, the powder merely being mixed to a paste and then shaken with the requisite quantity of water for 30 sec. The values obtained (39·5% at 20° C. and 57·5% at 50° C.) have been arbitrarily inserted on the zero ordinate in Fig. 1, and joined to the curves by dotted lines. It will be seen that the two points form possible origins for each of the two sets of curves, which would, of course, in any event be expected to intersect on the zero ordinate.

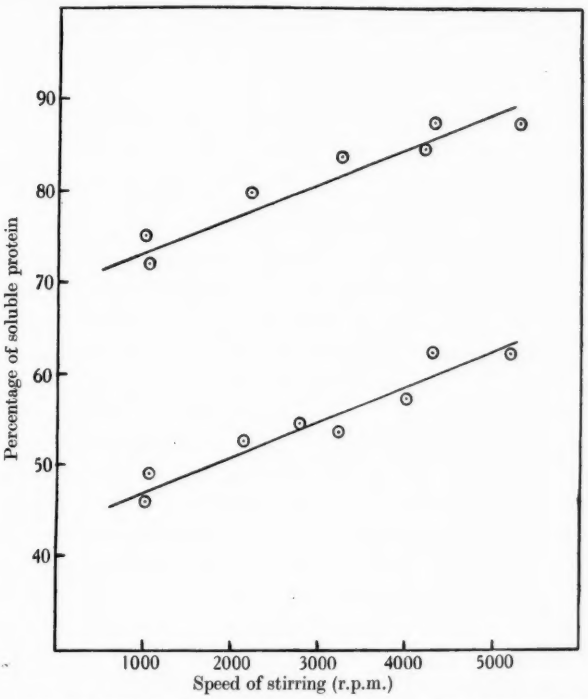

Fig. 2. Effect of speed of stirring on protein solubility; upper curve reconstituted at 50° C., lower curve reconstituted at 20° C. Duration of stirring 45 minutes.

The above facts show clearly that for any given temperature the solubility of a milk powder cannot be regarded as an absolute value which depends solely on the individual properties of the powder in question. On the contrary, the results indicate that, provided sufficient time is allowed for equilibrium to be reached, a different solubility level will be obtained for each speed of stirring employed. It may be noted, too, that the speed of stirring has a far more profound influence on the apparent solubility than the duration of stirring, although the latter is also a factor of obvious importance.

It was originally assumed that as the conditions of reconstitution became more drastic, the solubility would reach a maximum figure which would

depend solely on the individual properties of the powder and which could not be exceeded by increasing the speed or the duration of stirring. It is clear from Fig. 1 that there is, in fact, no such maximum value, and that if the speed and duration of stirring could be substantially increased, a solubility of practically 100% might possibly be obtained for almost any milk powder. It is somewhat difficult to reconcile this conclusion with the well-known fact that for practical purposes milk powders can be readily differentiated according to their relative insolubility, and that it has, in fact, been possible to demonstrate that this insolubility is of two types, a reversible type (in which the milk protein is capable of solution in hot water) and an irreversible type which is assumed to be due to true denaturation (Wright(1)). It is possible that the explanation of the results may be somewhat as follows:

It will be seen from Fig. 1 that for each speed of stirring the corresponding curves at 20 and 50° differ by approximately the same interval (i.e. 25–30%) throughout the entire range of times studied. It seems reasonable to assume that this difference represents the fraction of protein which is reversibly insoluble, i.e. which is insoluble in cold but soluble in hot water. Apparently the solubility of this fraction is unaffected by either the speed or the duration of stirring. If so, the marked differences in solubility illustrated in Fig. 1 must be associated with the irreversibly insoluble (i.e. the denatured) fraction of the milk protein. Presumably the high speed of stirring must produce a mechanical disintegrating effect on the particles of the denatured protein sufficient to reconvert (i.e. peptize[1]) them to a relatively stable suspension. In any such action it might be expected that the higher the speed of stirring the greater would be the disintegrating effect and that (as is shown in Fig. 2) the extent of disintegration and, therefore, of peptization of the protein particles would be roughly proportional to the violence of the stirring as measured by the stirring speed. The curves in Fig. 1 would, moreover, tend to be asymptotic, a fact which would account for the small effect caused by relatively prolonged periods of stirring.

While further work is required to determine the validity of this explanation, the present results are clearly of considerable practical importance in connexion with the formulation of standard methods for determining the solubility of milk powders, since it is obvious that the apparent solubility can be varied at will by altering the speed of stirring. Reference to Fig. 1 will show the magnitude of the differences which might thus be obtained. It will be seen, for example, that reconstitution of the milk powder for 30 min. at 50° C. using a speed of 4300 r.p.m. gave a solubility of 87%, while an equal period of stirring at a speed of 1100 r.p.m. gave a solubility of only 70%—a difference of 17% which is solely attributable to alteration in the speed of stirring. Although more prolonged periods of stirring reduced this difference somewhat, the fact that the curves run roughly parallel after about 100 min. indicates that the

[1] In the present paper the term "peptize" is given the broader meaning suggested by Bancroft(4), i.e. the dispersion of a substance to a sol.

figures will continue to deviate (by about 10%) however long the solutions are stirred.

In this connexion it should be noted that difficulties are frequently experienced in maintaining small electric motors (such as are commonly used for stirring) at a relatively constant speed. Alterations due to "warming up" of the motor and resistances and to slight temporary fluctuations in the electric supply may result in variations in speed of from 500 to 1000 r.p.m., and unless special precautions are taken to check the speed regularly, considerable errors will inevitably be introduced into the determinations.

Under these circumstances it would appear that, in devising any standard test for the determination of the solubility of milk powders, it is desirable to employ a technique which involves a minimum of stirring or agitation. Further reference to this point is made in Part II.

Part II. The relation between the solubility of the protein and that of the total solids

Throughout the work described in Part I, the solubility values were calculated on a protein basis on the assumption that the insoluble portion of a milk powder will consist almost entirely of insoluble protein. But as small amounts of other constituents might be rendered insoluble by the drying process and as the estimation of nitrogen requires much time and attention, it was decided to study the possibility of determining the solubility values on a total solids basis. For these reasons total solids were estimated in addition to nitrogen throughout the experimental work just described.

From the data thus available it is possible to determine the extent to which the solubility values calculated by the two methods agree. The milk powder used in this work contained 34% protein, 4% moisture, and therefore 62% of non-nitrogenous solids. If it be assumed that the insolubility of the sample is dependent on the insolubility of its protein, it should be possible to calculate the solubility of the total solids from the protein values. Thus in one particular determination where 75·0% of the protein was found to be soluble, the solubility of the total solids by calculation should be $62+\left(34\times\frac{75}{100}\right)$ or 87·5%. By total solids determination, the figure was found to be 86·5%. Examination of a series of forty-eight similar comparisons showed that the extreme differences between the calculated and actual values was 1·9% and the mean difference 1·0%, and that with only four exceptions the calculated values for the solubility of the total solids were found to be higher than the values arrived at by actual experiment. Such a finding supports the view that a small proportion of the non-nitrogenous constituents of dried milk are normally included in the insoluble fraction. The portion so included may be estimated at about 1% of the original powder or 1·6% of its total non-nitrogenous constituents. This figure agrees roughly with that given by Wright(1) for the non-nitrogenous

solids which are normally rendered insoluble by heat treatment during drying. It is clear, therefore, that while the protein solubility may be used to arrive at a fairly close approximation of the solubility of the total solids, the existence of a small insoluble fraction of the non-nitrogenous constituents will invalidate any attempt at an exact calculation. It may be pointed out, too, that it will also render even more difficult an exact estimation of the amount of soluble protein in a powder from total solids estimations. Thus in the instance already quoted (p. 503), the total solids dissolved were found to be 86·5% of the original powder which contained 4% moisture. The insoluble solids of the powder therefore amounted to 96 − 86·5 or 9·5% of the original sample. If this is assumed to be all protein it would give the protein solubility as $\frac{34-9{\cdot}5}{34} \times 100$, or 72·1%, whereas the actual figure was found to be 75·0%. There can be little doubt that the total solids method gives the more accurate value for the solubility of the powder as a whole. Further evidence for this is obtained by comparing the duplicate estimations of nitrogen and total solids.

The accuracy of duplicate determinations. All the solubility results to which reference has so far been made have been the mean values of duplicate and in some instances triplicate estimations of nitrogen or total solids. Data are therefore available from which the magnitude of the errors involved in individual determinations may be calculated and the relative accuracy of the protein and total solids methods compared. The results are summarized in Table I, where it can be seen that, with one exception (i.e. total protein at 50° and at 4300 r.p.m.), the maximum and the mean differences between sets of duplicates or triplicates were always greater for the nitrogen estimations than for the total solids.

Table I. *Differences between duplicate solubility percentages determined by both total solids and nitrogen*

R.p.m.	No. of estimations carried out in duplicate or triplicate at each temperature	Difference between duplicates	Total solids		Total proteins	
			20° C.	50° C.	20° C.	50° C.
1100	8	Maximum	1·50	1·00	3·60	3·10
		Minimum	0·00	0·20	0·60	0·00
		Mean	0·60	0·64	1·66	1·32
2200	8	Maximum	1·70	1·40	3·20	3·00
		Minimum	0·10	0·10	0·20	0·00
		Mean	0·85	0·58	1·76	1·37
4300	8 at 50° C. 7 at 20° C.	Maximum	1·40	2·40	4·60	1·10
		Minimum	0·00	0·00	0·00	0·10
		Mean	0·83	0·59	1·84	0·76

It had previously been assumed that an estimation of the solubility of milk powder on a protein basis would provide a more delicate indication of any variation in method of manufacture than an estimation based on the determination of total solids, since alterations in the protein would undoubtedly be the chief cause of insolubility. Thus a difference of 15% in the solubility of the protein (say from 67 to 82%) would correspond to a difference of only 5% in

the solubility of the total solids (i.e. from 85 to 90%). It is, however, apparent from the above discussion that although the protein method of estimating and expressing solubility will be preferable in studies concerned solely with the investigation of protein denaturation, the total solids method is preferable where a figure is required for the *total* solubility of a sample, as, for example, in industry. In this connexion it may be noted that the total solids method not only gives more reliable results but that it requires less apparatus and is far simpler to carry out in routine control laboratories than the protein method.

Part III. A rapid method for determining the solubility of milk powders

In any extensive series of determinations of the solubility of milk powders, such as is necessary in routine control work, the stirring process in the method previously advocated would prove most inconvenient, since it involves the use of an electric stirrer for a period of 30–45 min. for each individual determination. Moreover, it has already been pointed out that in devising a standard test for estimating the solubility of a milk powder a technique involving a minimum of stirring or agitation should be employed. It was therefore decided to try out a method in which the powder was mixed with water and simply shaken rapidly for a short period, the shaking being followed by centrifuging in the usual way, and the solubility figure being estimated on a total solids basis.

A rapid method for solubility determinations. 1 g. of the powder to be tested is weighed into a 15 ml. centrifuge tube. About 2 ml. of water are added from a burette and the mixture stirred well with a glass rod which had been previously wetted. When all the powder has become thoroughly moistened, more water is added until a total of 9 ml. has been run in, the stirring rod being washed with the last few ml. of water. The tube is then stoppered and kept in a water bath either at 20 or 50° C. for 5 min. and is then shaken rapidly for 1 min. The shaking speed will affect to some extent the solubility figure finally obtained, but if the process be carried out as vigorously as possible, involving some 4–6 complete double excursions per second, very close agreement is obtained by different workers for the same sample. If it is desired to estimate the solubility at 50° C. the tube is shaken inside a container lined with cotton wool to conserve the heat. The tube is then cooled to room temperature and centrifuged, the supernatant layer poured off as completely as possible (including the fat if the sample is a whole-milk powder), and its total solids content estimated by the rapid method of Golding (5). The ratio of the dissolved solids to the solids initially present (expressed as a percentage) is taken as an index of the solubility. The initial solids must be corrected for moisture content. Golding's method has been found most convenient and reliable provided phosphorus pentoxide be used as drying agent in the desiccators. Without such an efficient drying agent the results tend to be slightly high.

The solubility values by this process after correcting for the moisture content have been compared with those obtained by the longer stirring method for both roller- and spray-dried whole and separated milks and a typical selection of the results chosen at random are shown in Tables II and III.

Reference to Table II shows quite clearly that for spray-dried powders the

Table II. *The solubility values found for spray-dried powders by the long stirring method compared with those obtained by the rapid shaking method*

Description and source		Solubility at 20° C.		Solubility at 50° C.	
		Long stirring method	Rapid shaking method	Long stirring method	Rapid shaking method
		Whole milk			
Source A		99·4	98·9	99·3	100·0
		102·7	99·0	99·5	103·4
		100·5	102·0	100·0	102·8
		101·0	99·9	101·3	102·6
	Mean	100·9	100·0	100·0	102·2
Source B		101·6	100·3	101·6	102·3
		101·2	100·9	101·6	102·2
	Mean	101·4	100·6	101·6	102·2
Source C		86·2	88·4	100·0	97·1
		86·3	87·5	99·5	98·0
		85·3	90·5	98·2	97·0
	Mean	85·9	88·8	99·2	97·4
		Separated milk			
Source C		99·6	96·8	98·0	101·6
		98·3	96·7	98·9	99·3
		99·2	96·2	98·5	96·9
	Mean	99·0	96·6	98·5	99·3
Source B		101·6	101·5	101·2	103·5
		100·8	101·1	101·2	101·7
	Mean	101·2	101·3	101·2	102·6
Source A		—	102·2	—	101·7
		—	102·2	—	101·1
		—	101·7	—	102·5
	Mean	—	102·0	—	101·8

rapid shaking method gives results which are practically the same as those by the longer stirring process. This is particularly so with the whole and separated milks from sources A and B. With the samples from source C the differences are a little greater. Thus a figure of 85·9 by the long method for the whole milk at 20° is paralleled by one of 88·8 by the rapid method, whereas for the separated milk sample, the relative values are 99·0 and 96·6. The value obtained with this powder by the long method is therefore lower for the whole milk and higher for the separated milk than the corresponding values by the rapid method. Such divergences cannot, however, be considered sufficiently great to invalidate the rapid shaking process as a routine method for measuring the relative solubility of spray-dried milk powders, for it will be realized from the discussion in Part I that any method devised for measuring milk powder solubility is bound to give results of a purely relative nature, and that almost any milk powder will ultimately show an apparent solubility of 100% provided that the duration and speed of stirring are sufficient.

It will be observed that in Table II many of the solubility values exceed the theoretical maximum of 100%. The explanation appears to lie in the fact that, when applied to spray-dried powders, both the long and the short methods are liable to show errors of +2–3%. Observed values of over 100% may therefore be taken to indicate complete solubility. For this reason the

Table III. *The solubility values found for roller-dried powders by the long stirring method compared with those obtained by the rapid shaking process*

Description and source		Solubility at 20° C.		Solubility at 50° C.	
		Long stirring method	Rapid shaking method	Long stirring method	Rapid shaking method
		Whole milk			
Source D		88·0	88·6	97·0	97·5
		86·2	87·9	98·3	97·2
	Mean	87·1	88·2	97·6	97·3
Source A (sample 1)		84·4	80·5	98·6	94·3
		86·6	81·2	97·6	91·2
		86·0	78·2	99·2	89·9
		87·3	80·4	97·1	92·7
	Mean	86·1	80·1	98·1	92·0
Source A (sample 2)		86·2	78·7	100·1	88·0
		87·3	77·4	100·4	88·3
	Mean	86·7	78·0	100·3	88·1
		Separated milk			
Source D		79·4	80·9	93·4	91·9
		78·5	80·3	93·1	91·9
	Mean	79·0	80·6	93·3	91·9
Source A		81·7	78·2	97·1	91·9
		80·2	77·5	97·4	93·9
		81·7	75·2	97·7	91·8
		80·3	77·1	97·9	92·4
		81·7	75·3	98·2	94·5
		81·7	75·4	98·0	95·0
	Mean	81·2	76·5	97·7	93·3
Source E		80·5	74·4	91·2	82·7
		78·1	74·1	89·4	83·6
		79·8	74·8	90·8	83·4
		—	75·2	—	85·0
	Mean	79·5	74·6	90·5	83·7

solubility value is frequently referred to as the solubility index in the present paper.

Turning to the results set out in Table III for the roller-dried powders it will at once be seen that for the samples from sources A and E the rapid shaking method gives values which are considerably lower than those obtained by the stirring method. Thus values of 86·1 and 98·1 by the long process are paralleled by values of 80·1 and 92·0 by the rapid process. That this does not necessarily hold for all roller-dried powders is shown by reference to the sample from source D where figures of 87·1 and 97·6 are paralleled by 88·2 and 97·3 when stirring for 45 min. is replaced by a short period of shaking. It can only be concluded from the results shown in Table III, which are a random selection from numerous tests of a similar nature, that with roller-dried powders the new rapid method for estimating solubility on a total solids basis will *usually* give a value which is a few units lower than that obtained by the stirring process, but that with some samples the values by the two methods will be practically identical. It seems probable that with some roller-dried powders the denatured portion of the protein does not readily become peptized even after prolonged stirring, and that consequently the stirring method and

the rapid shaking method both give similar results. With other powders 45 min. stirring may be sufficient to peptize a small amount of the insoluble protein although 1 min. shaking fails to accomplish this. If this is the true explanation the rapid method should actually provide a more accurate index of the solubility of such samples than the longer process. It may be noted that the rapid method has been successfully employed at this Institute in recent large-scale investigations into problems connected with the storage of milk powder, the details of which will be reported at a later date.

Part IV. The estimation of the solubility by the sediment method

A simple method which is commonly employed in industry for determining the solubility of dried milk is the reconstitution of the sample in a 10% aqueous solution and the measurement of the volume of sediment after centrifuging in a graduated tube. During the course of the present work the opportunity has been taken to compare results obtained by the new rapid method with those obtained by the sediment method.

Comparison of the new rapid method with the sediment method

In Fig. 3 the sediment volumes of sixty-five different samples of dried milk are plotted against the solubility indices determined by the rapid method. It will be seen that the points fall in a fairly wide band along a smooth curve. The sediment volume may therefore be taken to provide a rough guide to the solubility of a milk powder. The limitations of the method must, however, be clearly recognized. For example, two roller-dried samples, each of which showed a solubility index of 85·2, gave sediment volumes of 1·98 and 2·45 ml. respectively. Two other samples, each of which gave sediment volumes of 2·4 ml., showed solubility indices of 72·8 and 82·5. Again, six samples, all giving sediment volumes of 2·0 ml., showed solubility indices of 77·7, 83·9, 84·3, 85·9, 86·9 and 88·2. These serious discrepancies occur chiefly in the lower half of the curve, where the slope approaches the vertical and where, therefore, changes in the solubility index are in any event accompanied by relatively small alterations in the sediment volume. Thus it will be seen from Fig. 3 that a change in the solubility index from 75 to 85 is paralleled by a reduction of only 0·35 ml. in the sediment volume (i.e. from 2·55 to 2·20 ml.), whereas a change in the solubility index from 85 to 95 is paralleled by a reduction of 2·0 ml. in the sediment volume (i.e. from 1·15 to 2·15 ml.). A discrepancy of 0·5 ml. between the theoretical and observed sediment volumes would therefore entirely mask differences in solubility of from 10 to 15% where the powder is of poor solubility; on the other hand a similar discrepancy would not be a source of serious error with more highly soluble powders. Indeed with powders of exceptionally high solubility (such as spray-dried powders) the sediment volume changes so rapidly with alterations in solubility that it may prove a most valuable confirmatory test. For example, in one experiment a milk

powder was submitted to treatment which was expected to alter its solubility only to a slight extent. Determinations made by the rapid method showed that the solubility index had fallen from 101 to 98, a difference of doubtful significance. Measurements of the sediment volume showed, however, a four-

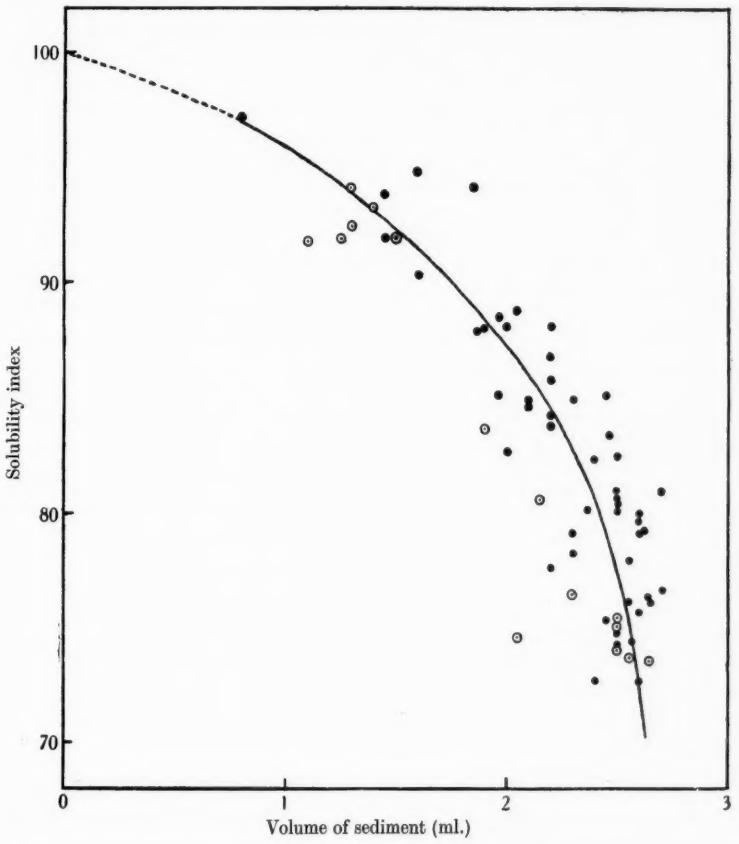

Fig. 3. The relationship between sediment volume and the solubility of roller-dried powders.

—●——●— Whole milk powders. —⊙——⊙— Separated milk powders.

fold increase from 0·1 to 0·4 ml., which provided clear confirmation that the solubility of the powder had in fact been altered as a result of the treatment.

It may, therefore, be concluded that the sediment method can supply valuable information regarding the comparative solubilities of powders which are normally highly soluble, such as spray-dried powders. The method is of very questionable value when applied to powders of relatively low solubility, such as those produced by atmospheric roller or drum drying.

The effect of repeated washings on the volume of sediment

It has been suggested (6) that the accuracy of the sediment method might be improved by pouring off the supernatant layer after centrifuging, shaking the sediment with a second volume of water, and recentrifuging. It was felt desirable to investigate this point.

1 g. of a roller-dried skimmed milk from source D was weighed into each of eight graduated centrifuge tubes and reconstituted at 20° C. with 9 ml. of water according to the rapid method for determining solubilities. After centrifuging, the volume of sediment was noted and one tube was used for determining the total solids content of both the supernatant layer and of the sediment. From the other seven tubes the supernatant liquid was removed, water added up to 10 ml., the mixture shaken for 1 min. and then centrifuged. Again one tube was used for the estimation of total solids content of the supernatant liquid and the sediment. This process was repeated until all eight tubes had been used, so that the sediment in the last tube had been washed seven times after the first centrifuging. The experiment was repeated with a second sample of roller-dried powder obtained from source A. In order to determine the effect of rapid stirring as distinct from shaking, the experiment was repeated on the first powder (source D) using 45 min. stirring. In this instance it was not found possible, however, to record the volumes of the sediment.

The results from these two powders, which show excellent agreement, are illustrated in Figs. 4–7. It will be seen (Fig. 4) that the total solids content of the supernatant liquid at successive washings falls along a smooth curve from about 8·5% (the solids content of the original reconstituted and centrifuged sample) to less than 1% after four washings. Fig. 5 shows a parallel fall in the total solids content of the sediment from 20% after the initial reconstitution to roughly 10% after four washings. Thereafter both curves remain at an approximately constant level. (It may be noted that the apparent rise in the total solids content of the sediment at the later washings in Fig. 5 is probably attributable to experimental error involved in estimating accurately the very small quantities of sediment present.) It was thought possible that the initial rapid fall in both curves might be associated with the "washing out" of that portion of the supernatant liquid which occupies the interstices between the insoluble particles of the powder, while the remaining small quota (indicated in Fig. 4 by the relatively constant level of the curve after the fourth washing) might represent a small but constant peptization of the insoluble particles themselves. Calculations showed that this assumption was correct. It was found that the weight of solids in the interstitial liquid (i.e. in the liquid between the insoluble particles) after the initial reconstitution was 0·160–0·180 g. for both powders. This value fell at successive washings to 0·035–0·050, 0·015–0·025 and 0·007–0·014 g. After the fifth washing the value was less than 0·002 g. On the other hand, the weight of solids composing the insoluble portion of the sediment after the initial reconstitution was between 0·180 and 0·260 g. and this value fell relatively slowly and at a roughly constant rate throughout the eight successive washings, the value at the final washing still being relatively high (i.e. 0·075–0·080 g.). This gradual decrease is shown

diagrammatically in Fig. 6, where it will be seen that the weights of the insoluble portion of the sediment after successive washings fall along two roughly straight lines.

The above calculations indicate that, while the total solids content of the initial sediment consists of roughly equal portions of soluble and insoluble

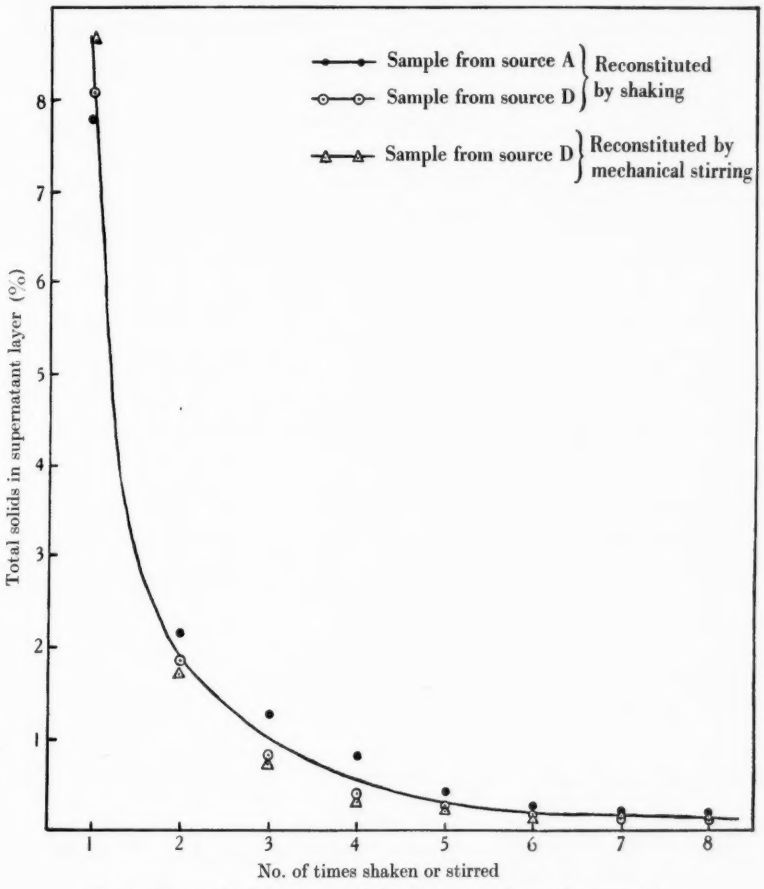

Fig. 4. The total solids content of the supernatant layer after successive shakings or stirrings.

matter, that of the final washings contains about forty times as much insoluble as soluble matter. This shows clearly that the major effect of washing the sediment is to replace the interstitial liquid with water.

On the other hand Fig. 6 shows that a portion of the insoluble matter is also peptized by washing, with the result that there is a constant reduction in the weight of the sediment throughout successive washings. This fact is confirmed by reference to Fig. 7, where the sediment volumes after successive

washings are plotted. The reductions in volume run remarkably parallel to the reductions in weight, both being reduced to about 30–50% of their initial values by the eighth washing. It has been found, moreover, that while with some powders washing causes a considerable decrease in sediment volume, with other powders the change in volume may be much less. Thus, of two samples which showed an initial sediment volume of 2·7 ml., one gave a sediment volume of 2·4 ml. after a single re-washing, while the other showed no change

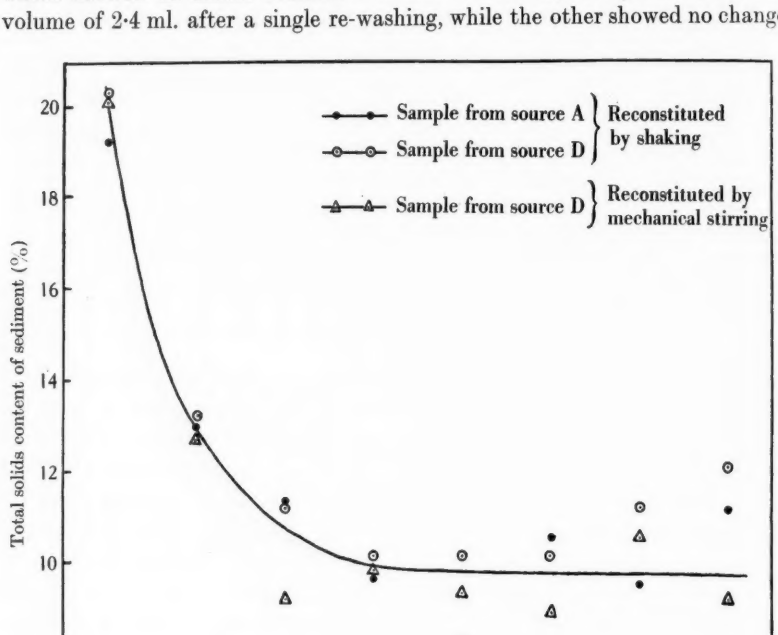

Fig. 5. The effect of successive shakings or stirrings on the total solids content of the sediment.

in volume. These facts provide a strong argument against the inclusion of one or more additional washings in any standard method for determining the solubility of milk powders.

It might, however, be urged that washing will be advantageous in causing the insoluble material to form a more compact sediment and in enabling the boundary between the sediment and the supernatant liquid to be more easily seen. Actually neither of these arguments is tenable. As regards the compactness of the sediment a comparison of Figs. 6 and 7 shows that the weights and volumes of the insoluble material run strictly parallel, and that the observed reductions in volume with successive washings are attributable largely—if not solely—to reductions in the quantity of insoluble sediment lost through

peptization and not to the formation of a more compact layer. As regards the clearness of the boundary between the sediment and the supernatant liquid, observations on a large number of samples of dried milk derived from different sources indicate that this is connected with the physical and possibly the chemical nature of the powder rather than with alterations in the nature of the

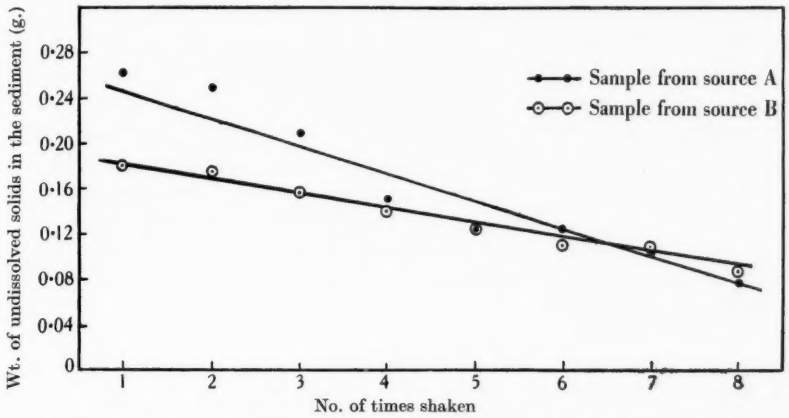

Fig. 6. The effect of successive shakings on the undissolved solids in the sediment.

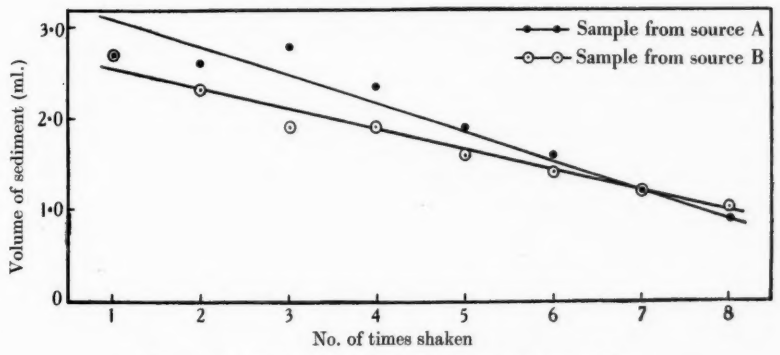

Fig. 7. The effect of successive shakings on sediment volume.

supernatant liquid. Thus powders which give compact and easily differentiated sediments after initial reconstitution and centrifuging usually exhibit the same properties at subsequent washings, while the loose and indefinite sediments given by other powders frequently show no marked improvement in this respect until they have been subjected to three or more washings—a procedure which would involve excessive peptization of the insoluble sediment and which would in any event be impracticable under commercial conditions.

SUMMARY

1. The effects of alterations in the duration and speed of stirring on the apparent solubility of milk powders have been investigated. It is concluded that 30 sec. shaking of the powder reconstituted in a 10% mixture is sufficient to dissolve the truly soluble portion of the dried milk, but that the protein which has become denatured during the drying process tends to pass into solution with increasing speed and duration of stirring.

2. A rapid method for estimating the solubility of milk powders on a total solids basis involving only 1 min. shaking is described and discussed.

3. The sediment method frequently used in industry as a guide to the solubility of dried milks is compared with the rapid method described in the present paper. At higher solubilities the sediment volume may serve as a guide to small changes in solubility, but in the lower solubility ranges its usefulness is very limited. It is shown that the washing of the sediment with one or more changes of water merely introduces additional errors into this method of solubility determination.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

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SECTION B. BACTERIOLOGY AND MYCOLOGY APPLIED TO DAIRYING

I. Milk control
Technique
II. Micro-organisms in milk and milk products
(*a*) *Coli-aerogenes* group
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I. MILK CONTROL

Technique

Since the introduction in January 1937 of the modified Methylene Blue Reduction test (Memo 139/Foods) as a measure of the bacteriological quality of producers' milk there has been a veritable spate of papers from this country and from America. The abandonment of the plate count was in the nature of a revolution and caused considerable perturbation. With the issue of Memo 139/Foods, 1937, which gave details of the technique for the examination of "graded" milk samples, vociferous criticism arose. Some who used the methylene blue reduction test officially published their experiences(1,2,3) which showed in the main that the results of the examination of a series of samples by this method were much the same as when the plate count was used. This was to be expected, but several authors have confirmed the results on which Memo 139/Foods was based by showing that the correlation coefficient "r", log of plate count v methylene blue reduction time, was in the range 0·80–0·85 in different experiments. It is interesting that Sandberg(4), using data obtained in 1912 and 1917 (when the old form of the methylene blue test was in use), finds very similar values of "r". Mattick(5) has estimated that in a series of three examinations (a series of examinations is of course the essence of any biological test), the chances of disagreement in all cases between plate count and methylene blue test were 1 : 487. But there is some evidence that the correlations are not as high in winter as in summer(5), suggesting that the methylene blue test may be influenced by factors not affecting the plate count. It was suggested that this was in part a question of "activity" of the bacteria in milk. Activity for our purpose may be defined as ability to initiate reproduction quickly and to produce substances capable of contributing actually or potentially to the reduction of methylene blue. It has been shown that organisms vary inherently in their reducing capacity(6) and therefore in their

power to spoil milk. It may therefore be argued that the methylene blue test which measures activity as well as, in some degree, numbers, is more valuable than the plate count which measures only numbers, and these inaccurately.

The reduction test, like the plate count, has been regarded as a measure of the excellence of the methods of production at the farm. But it has long been known that no adequate measure of production conditions can be got from bacteriological examination, by any known means, of milk examined at once or within a very few hours of production (7). It is conceded by all that a period of "incubation" is necessary before the milk is examined. Under these conditions, at least some reflexion of conditions of production at the farm may be secured. Such a procedure rests on the basic bacteriological fact that the extent of growth in a given time under given conditions is a function of the numbers originally present, i.e. the greater the numbers initially present the greater will be the growth and the less the lag period—influenced in milk by the bacteriostatic and other properties (6). In other words, well-produced milk preserves a low bacterial count much longer than badly produced milk.

But when one tries to measure the correlation between the factors contributing to the conditions of production at the farm and the results of methylene blue tests and plate counts the value of the coefficient "r" is found to be unexpectedly low (8, 9). It is, therefore, likely that many small chance factors influence the result, and one is not likely to estimate their effect accurately by inspection or even by detailed examination of any factor known, in general, to contribute to the result, e.g. swabbing of utensils and bacteriological estimation of the numbers. There is no doubt that the temperature to which samples are exposed and the time of exposure are in the main overpowering factors in determining the result of a bacteriological examination of the milk. This fact ought to be generally known, but it is surprising how often it is neglected. In Memo 139/Foods it is laid down that milk shall be subjected to certain periods of exposure to "atmospheric shade temperature" before examination, presumably on the assumption that good milk may justly be expected to withstand reasonable exposure to atmospheric temperature. But milk cannot reasonably be expected to endure indefinitely temperatures "in the 70's" in an English summer. Conversely, in winter when temperatures are low there will be insufficient growth to distinguish badly produced milk from well-produced milk. These facts cut at the roots of the assumption, on which grading is regarded by many, that a methylene blue test or a plate count gives a more or less accurate reflexion of production conditions. Mattick (10), in a preliminary report of the results of a co-operative experiment, quotes figures which show that the value of the correlation coefficient, plate count *v.* methylene blue reduction test, is high in summer and low in winter, tending to confirm the statement that temperature (with time) is an important factor in determining the bacterial count. Barkworth (11) has therefore proposed that samples for examination be transported in insulated boxes with the object of preventing, in summer, the exposure of milk to excessive temperatures. Alternatively, Hoy & Braz (12) have suggested that milk incubation for a fixed time at a fixed temperature should be in the hands of the bacteriologist. There are obviously practical difficulties in either of these suggestions, but some reform in the management of samples prior to examination seems to be necessary if the result of a bacteriological examination is in fact to justify the common belief that the conditions of production on which a bonus for clean milk is paid are measured by such an examination of the milk.

Nothing which has appeared in the literature during the period covered by this review has, in our opinion, done anything to refute the opinion that the methylene blue test is well adapted to its purpose. It is indeed possible that some adjustment of the methods of management of samples, of the standard reduction times and even of some details of the technique may be desirable, but none of these detracts from the

basic value of the test for its purpose. As an example of the precautions which may be necessary in the management of the solution of methylene blue itself under routine conditions, Mattick & Neave (13) report that growth of organisms derived from tablets may actually take place in a methylene blue solution. Incidentally, attention has been drawn to the fact that the final concentration of the dye in a sample for examination is 1/550,000 and not 1/300,000 when "standard" (Danish) tablets (and solutions) of methylene blue are used (6).

The rapidity and cheapness of the methylene blue test (always bearing in mind that the cost of examination of a sample is the cost of collection + laboratory examination (1)) has encouraged the exploration of the possibilities of *resazurin* reduction as a measure of milk quality. This substance is an oxidation/reduction indicator similar to methylene blue, but reduction to the white stage takes place in two stages. Visible change in the blue colour takes place at a higher E_h than methylene blue. Several authors (14,15,16,17) have reported on the use of resazurin, and on the whole their conclusions are not unfavourable. It was to be expected that, since it is more labile than methylene blue, this dye would tend to be affected by weak reducing systems, not necessarily bacterial. Thus the first stage of reduction appears to be of a chemical rather than of an "oxidation/reduction" (reversible) nature. Nevertheless, by taking advantages of the gradual nature of the colour change, it appears, in spite of the fact that its value for grading is not yet clear, that as a means of sorting milk which is suitable from that which is unsuitable for a factory process, e.g. cheesemaking, the resazurin reduction test may be of value, if only because of its rapidity. Davis (18), for example, has shown that it is possible to secure a result within a few minutes.

Although the plate count (except for pasteurized milk) has been abandoned for official grading purposes in England it still preserves (as we pointed out in a previous review) its value as an advisory test. Although in America the use of the methylene blue test, even in its original form, has been permissive it appears from the large number of papers which have appeared in the last two years that the plate count will continue to be used there.

The earlier work showing the improvement in the old American Standard Agar which results from the addition of milk has been accepted in America (19), and the value of various other additions such as tryptone and dextrose have been appraised. The controversy as to what shall be the standard temperature of incubation still continues, and Yale has examined many figures for counts at 32 and 37° C. (20). There is no doubt that at say 30 or 32° C. the bacteria which commonly occur in numbers in milk and play a great part in determining the keeping quality grow better than at 37° C. It seems probable that some temperature lower than 37° C. will be adopted in America, although no change in the incubation temperature is made in the new (7th) edition of American Standard Methods. One may again deplore the complication of composition entailed by the use of numerous ingredients. Anything tending to simplification consistent with reasonable accuracy is surely to be aimed at when standard methods are in question.

After a long period of stasis the use of paper-board cartons for milk distribution appears to be gaining ground in some parts, at least, of America (21) and for special types of trade in England (22). It has been urged that, because of their weight and the vigilance necessary to ensure the proper performance of many mechanical washing processes, cartons might well replace glass bottles if the factor of cost can be met. If cartons are to be used for liquid milk it is of course essential that they shall be as free from organisms as possible. This aspect has received consideration in America and numerous publications have appeared. These deal with the factors governing bacterial and mould growth during manufacture of the paper board, including the purity of the process water, the cleanliness of the mill operations, and the protection from con-

tamination of the finished product. The New York State Agricultural Experiment Station has devoted a department to the study of the bacteriological condition of paper for use with perishable foods and to the establishment of standards for carton examination, and Sanborn (23,24) has summarized the present state of the work. He states that with paper board made under good conditions containers can be practically free from micro-organisms, and Tanner (25) found that the counts obtained with cartons are generally lower than those with glass bottles. The flora consisted mainly of cocci, aerobic spore-formers and non-spore-forming rods. Good methods of manufacture and hot paraffining markedly reduce the flora. An important point in Tanner's work is that the cartons giving such favourable results were made in the factory. It is evidently no longer necessary to advocate the shaping of containers in milk depots immediately before use (26).

It has been proposed that the paper board shall be disintegrated mechanically before bacteriological examination (27,28), and that large volumes of the fluid used for rinsing finished cartons shall be plated to determine surface contamination (26).

Considerable interest continues to be shown in the bacteriological condition of glass bottles, and the new (7th) edition of American Standard Methods (19) for the examination of Dairy Products lays down a standard method for glass bottles. It has often been shown, lately by Mattick & Hoy (29), that bottles leave the detergent section (detergents having replaced other methods of sterilization in large installations) in a virtually sterile condition but are liable to heavy contamination by rinse water in the later stages. These workers showed that if temperatures of working are properly maintained lower strengths of alkali than were formerly demanded suffice to yield satisfactory bottles. On the other hand, if the minimum strength of alkali is not maintained throughout the whole time of working, glass bottles may be dangerous, as Mattick & Hoy found that in badly managed commercial machines haemolytic streptococci could be recovered from bottles which had passed through. These workers showed that faults in construction contributed largely to the failure of some machines to provide satisfactory bottles.

There is always danger in setting up numerical standards, but American Standard Methods, 1939 (19) recommend a maximum of 1000 colonies per quart (American) bottle and of 500 per pint bottle as a measure of proper cleaning. Hoy & Mattick (29), who recognized that the relationship of "volume to area" of bottle should be taken into account, suggested tentatively that 200 organisms per pint bottle should be the standard. American Standard Methods admit that properly sterilized bottles will meet more severe standards than those they propose.

II. MICRO-ORGANISMS IN MILK AND MILK PRODUCTS

(*a*) *Coli-aerogenes* group

At first sight it would almost appear that efforts to extend the knowledge of this group of bacteria leads only to greater confusion. The importance of these organisms cannot be denied, for although they cannot actually be regarded as pathogenic their presence in water supplies, milk supplies and foods always implies uncleanly conditions. The conception of the significance of these organisms varies with the point of view; the hygienic aspect is not identical with the economic. For this reason lines of research vary, and the results accumulated during the relatively short period of this review appear to consist mainly of isolated or contradictory facts. Even the nomenclature is confused, due in part to popular interest in this group and the consequent need for terms less formidable than, for example, "Escherichia-Aerobacter". The

need for a common terminology applying equally to the results of examination of water supplies, milk supplies or milk products, has been stressed by Breed & Norton (1). In a review of this group of organisms as they occur in dairy products, Yale (2) has discussed their relative significance in water supplies and dairy products and has commented on the value of the various quantitative and qualitative tests.

In some circumstances it is important to be able to distinguish between and even to estimate numerically the various members of this group. The search for the ideal selective medium and for reliable tests distinguishing between the various groups still continues. Ritter (3) advocated an increase of the basic fuchsin content of fuchsin-lactose broth when used as a confirmatory medium in water analysis. On the basis of the examination of 1108 samples of water, 550 of which contained coliform organisms, Bardsley (4) concluded that the confirmatory test described by Wilson *et al.* was to be preferred to the English Official Method used in water analysis. McCrady (5) studied six selective methods for the detection of coliform organisms in water and found that the brilliant-green-bile confirmatory method was the most generally satisfactory. He suggested that this might with advantage replace the American Standard Method. Gunsalus & Stark (6) found the use of formate-ricinoleate broth to be equal to the Standard Method for the detection of the *coli-aerogenes* group in raw and pasteurized milk, and Littman & Stark (7) were able to differentiate these organisms by colony form and to estimate their numbers on citrate-ricinoleate agar.

The importance of methods of detecting the presence of coliform organisms was first realized in connexion with the analysis of water supplies. It was only later that these methods were extended to the examination of milk and milk products, and the direct application of the methods used in water analysis has led to misconception. The danger of a procedure which has made no allowance for possible variation in the reaction due to the composition of the medium under examination has been emphasized by McAuliffe & Farrell (8). One ml. portions of milk adsorbed a considerable quantity of the dyes contained in selective media, the inhibiting action of which was thereby lessened. They estimated that the content of basic fuchsin in fuchsin-lactose broth must be increased seven times, and the brilliant green dye in brilliant-green-lactose-bile broth $2\frac{1}{2}$ times if the selective action of the medium is to be retained. This danger was also recognized by Yale (9), who concluded that of ten media examined, desoxycholate agar gave the best all round results for pasteurized milk. He advocated the use of the plate-count method only where it is desirable to follow up results of qualitative tests.

The value of the use of Eijkman medium at 46° C. in differentiating coliform organisms isolated from milk has again been demonstrated by Bartram & Black (10). The favourable action of buffers on gas production by various species of *Bact. coli* at 37 and 46° C. has been shown by Hajna (11). Davis (12) concluded that brom-cresol-purple was superior to litmus as an indicator in the "presumptive *coli*" test. It is now generally recognized that the chief value of the "presumptive *coli*" test for pasteurized milk is that it may be taken as evidence of post-pasteurization contamination. This is also true for cream and the butter made from it (Rice (13)). Although, as has been demonstrated by Elliker & Frazier (14), the resistance of *Bact. coli* varies and is highest at the maximum stationary phase of growth and lowest during the period of most active growth, true thermoduric strains which would be present in the milk as it leaves the holder are comparatively rare. MacLean (15) suggested that daily routine testing with brilliant-green-bile broth, the plate count being reserved for occasional supplementary tests, would provide a satisfactory means of controlling the efficiency of pasteurization. Experimental data on the determination of the *coli*-titre by the dilution method have been given by Matuszewski & Supinska (16). Since the time factor is a matter of importance in the control of pasteurization plants, the

results obtained by the use of violet-red-bile agar as suggested by Miller & Prickett (17) and the brilliant-green-bile-salt lactose-peptone-water as used by Kraievskaya (18), both of which gave satisfactory results in 22–24 hr., may be of interest.

The attempt to establish the type of coliform organisms that are dominant in the various grades of milk and milk products has not yet been successful. Bartram & Black (19) examined 390 samples of raw, pasteurized and certified milk. They found a great variety of all types, none of which was specifically characteristic of any designation of milk. They suggested that the coliform group as a whole was more significant than any one genus in indicating contamination. The findings of Stuart *et al.* (20) for eighty samples of certified milk and forty samples of milk of relatively poor quality were very similar. Their results led them to doubt, with others, the value of the coliform count in assessing the hygienic condition of milk.

Workers in this field find difficulty in comparing results because of the lack of a generally accepted scheme of classification of this important group of organisms. The limitation is fully realized, but although much work is being done in this direction there seems to be a tendency towards over-classification and multiplication of difficulties rather than towards simplification of the whole scheme. Malcolm (21) has found it possible to arrange 1636 cultures isolated by him from milk and bovine faeces into eight well-defined subgroups by means of the Voges-Proskauer, Koser, inositol and indol reactions. Parr (22) used the indol, methyl-red, Voges-Proskauer and citrate utilization tests as the basis for his studies. He was of the opinion that the classification of this group should be simple and might be achieved within four species of the genus *Bacterium* to include *Coli*, *intermediate aerogenes* and *cloacae*. Stuart *et al.* (23) examined 3247 strains and divided them into three major groups, Aerobacter, Intermediates and Escherichia on the basis of indol and Voges-Proskauer tests. On the basis of serological tests Bredenbröker (24) established twelve groups. No correlation was demonstrated between serological and biochemical tests. Staberow (25) has studied the morphological and physiological characters of sixty typical and atypical coliform strains, and Oeser (26) has examined 164 strains frequently found in milk, by means of numerous tests, the value of each of which he discussed. The possibility of placing too much reliance on biochemical reactions in schemes of classification is suggested by the numerous instances of the relative ease with which variants and mutants arise in cultures cited by Nyberg *et al.* (27,28), Sievers (29), Tregoning & Poe (30), Sherman & Wing (31) and Jéline & Rosenblatt (32). The Voges-Proskauer test is usually regarded as a good and consistent test distinguishing between the *coli* and *aerogenes* groups, but Nyberg *et al.* (27) found that even in this respect some strains were not entirely stable. Tittsler (33) also found that approximately 50% of his strains of *Bact. aerogenes* and *Bact. oxytocum* were able to ferment acetyl-methyl-carbinol and thus gave negative Voges-Proskauer tests after 3–5 days' incubation in Clark & Lub's medium. He suggested that the Voges-Proskauer test should be carried out on 2-day-old instead of on the usual 5-day-old cultures.

Lipska (34) has given details of the technique used in her examination of 105 strains of coliform organisms for sensitiveness to bacteriophage. She found that the sensitiveness varied inversely as the rate of growth at 32 and 37° C. and the fermentative activity. The sensitive strains were characterized by great constancy of properties. Schioppa (35) found *coli* bacteriophage in none of thirty samples of milk from different farms, but the addition of a phage isolated elsewhere brought about lysis of added coliform organisms, from which he concluded that lysis is not impeded by milk constituents.

(*b*) Spore-bearing bacteria

The importance of the spore-bearing bacteria, in view of their frequent occurrence in milk products, is evidenced by a number of papers dealing specifically with them. The anaerobic spore-bearers are of particular interest in various canning processes. A hindrance to the ready study of anerobic organisms is the difficulty of culture, and efforts are continually being made to simplify the procedure. Two such attempts at simplification have been put forward by Müller (36) and Koschucharoff (37). A study of the morphological and physiological properties of a group of butyric acid-producing anaerobes has been made by Sjolander & McCoy (38), while Dimitrijević-Speth (39) has described the separation of non-motile from motile forms by means of streak cultures in semi-solid agar. Porter *et al.* (40) have studied the facultative anaerobic spore-bearing bacteria producing gas from lactose. They distinguish two types, *Aerobacillus polymyxa* and *A. macerans*. A comprehensive review of anaerobic bacteria in general has been given by Weinberg *et al.* (41).

The economic importance of the spore-bearing bacteria depends to a very large extent on the marked resistance of the spores to heat. The mechanism of spore production and the real understanding of the reasons for the high resistance of the spores still offer a very wide field for research, to which several contributions have been made. Recent determinations of the water content of bacterial spores and vegetative forms suggest that, contrary to views previously held, there is no essential difference in this respect between the two types of cell, and that therefore low moisture content cannot be the explanation of the high resistance of spores. This question has been studied in some detail by Henry & Friedman (42,43) for *B. subtilis*, *B. megatherium* and *B. mycoides*. Special attention was paid to the "bound-water" content, and they found that in all cases the spores had a greater water-binding capacity than vegetative cells. They suggest that the heat resistance of bacterial spores is due, in part at least, to their relatively high "bound-water" content. Von Angerer (44) has carried out extensive researches on the influence of the various factors, such as the fat content and the adsorption of the spores on porous substances, but no very clear conclusion seems to have been reached. It has often been suggested that the resistance of spores as determined in the laboratory, using artificial cultures, is not necessarily a measure of the resistance of spores as they occur in nature, and therefore in the state in which they often invade food products. Görtzen (45) did not, however, find that "native" bacterial spores, tested directly from sixteen samples of soil, were significantly more resistant than the spores of the same strains of bacteria after artificial cultivation in the laboratory. Nevertheless, variations in the heat resistance of spores, sufficient to affect the processes in the food-canning industry, have been recorded. In this connexion Townsend *et al.* (46) have noted that the resistance of spores of *Cl. botulinum* in canned foods is different from that in neutral phosphate solutions. Their results indicated that the resistance was essentially the same in whole milk and in skim milk but greater in cream. The temperature of incubation of the trial culture appeared to influence the result. This aspect of the problem has also been studied by Theophilus (47) for organisms isolated from spoiled and normal evaporated milk. He found that the thermal resistance of the spores was greatest from cultures grown at the optimum temperature. Growth for even one generation at a favourable temperature increased the resistance of the spores. He suggested that a period of high temperatures may therefore explain sudden outbreaks of trouble in the evaporated milk industry, and used this as an argument in favour of keeping milk at low temperatures from the time of production to the time of processing, as a preventive measure.

III. LACTIC ACID AND ALLIED FERMENTATIONS

(*a*) Bacterial metabolism

General

A second edition of M. Stephenson's well-known "Bacterial metabolism" (1) has appeared, and C. G. Anderson has written an introduction to bacteriological chemistry (2) which covers a new field. A monograph on the influence of cations on bacterial growth (3) has been written by L. Neipp, and a short review of recent work on the biochemistry of bacteria comes from van Niel (4).

Of papers dealing with technique of interest to dairy bacteriologists, mention may be made of Friedemann's detailed description of methods in metabolism studies (5) and of a simple method for measuring gas production devised by Allen (6). White & Ward have described a tube for the cultivation of gas-forming anaerobes (7) which avoids contamination of the plug.

Lipmann's review of biological oxidation and reduction (8) deals with many systems of importance in dairy bacteriology, and Clark (9) has listed E_0 data for many of these. The oxidation-reduction systems in milk have been studied by Twigg (10), who has made many observations which are of both fundamental interest and practical importance in relation to the methylene blue test. He found that cream reduced methylene blue easily under anaerobic conditions but that washing removed the activity, which could be restored by adding heated milk. The fat-globule membrane substances plus this "inactive fraction" also reduced the dye, the former containing a thermolabile enzyme. Pasteurized whole milk would not reduce, whereas autoclaved skim milk reduced methylene blue. Of possible activators for reduction, hypoxanthine was the most efficient.

The fundamental investigations of Meyerhof (11) on the lactic and alcoholic fermentations and of Werkman *et al.* (12,13,14,15) on the propionic acid fermentation have been continued but cannot be discussed here in detail.

Protein and fat metabolism

Controversy continues on the question of the mechanism of proteolysis by bacteria, and the respective roles of extracellular enzymes and living cells. Virtanen & Suolahti (16) hold that degradation is due entirely to the enzymes excreted from the cells; this liberation of enzymes may reach a maximum in 10 hr. Gorbach & Pirch (18) oppose these views and have made certain criticisms of published work, pointing out, amongst other things, that casein is not a suitable substrate for *Ps. liquefaciens*. Virtanen & Suolahti (17) have reaffirmed their statements and claim that the activity of the cells is practically nil, and that this holds both for casein and gelatine. Gorbach (18) has studied further the enzymic systems of Gorini's acido-proteolytes. These show maxima at *p*H 7 and also at *p*H 5–6. The purified proteinase is most active at *p*H 4·7 and is stimulated by KCN (cf. papain); the peptidases show two optima at *p*H 4·8 and 8·4. Console & Rahn (19) find that proteolysis by *B. subtilis* is due partly to extracellular enzymes, and that the presence of glucose in the medium inhibits the formation of the enzyme.

Sudhaus (20) has completed a detailed study of the factors controlling the activity of rennet-forming bacteria. He found that the alkali-forming rods were more active at lower temperatures, but that acid and alkali had little effect. Sugar reduced the rennet activity in old cultures. The optimum temperature for production of rennet by the cocci was 37° C., although the maximum temperature for the enzyme was 70° C. (cf. the plant rennins).

Long(21) has studied the lipolysis induced by organisms isolated from dairy products and found that Nile blue sometimes inhibits lipolysis. He states that *Str. lactis* hydrolyses tripropionin and tributyrin but not cotton-seed oil or butterfat. Jensen & Grettie(22) came to the conclusion that no medium was really satisfactory for the detection of lipolysis but found that a modified form of Turner's Nile-blue-cotton-seed-oil agar was best. From the point of view of food deterioration, lipase-oxidase producers are more important than those organisms producing lipase only. They found that moisture-free fat did not support growth but that 0·3% moisture was sufficient to permit growth. Trussell & Weed(23) have found that staphylococci possess a considerable lipolytic activity but could obtain no correlation with virulence or assistance in classification.

Clark & Tanner(24), continuing their studies in the sulphur metabolism of micro-organisms, found that peptones varied greatly in their ability to reveal formation of H_2S, Witte, Wilson and Parke Davis peptone being the best. Thermophilic organisms could rapidly split H_2S from cysteine but not from methionine, and that although all liquefied gelatine, only a few produced H_2S. Sulphur, thiosulphates and sulphites were rapidly reduced to H_2S.

Nutrition

Lwoff(25) has written a review of the growth factor requirements of micro-organisms which is very readable and up-to-date, although some of the formulae are given incorrectly. Koser *et al.*(26) report that some gelatines have a growth factor effect which is removed by purification, and Koch(27) finds that yeast preparations are as good as meat extracts for pigment-forming organisms. Curran & Evans(28), studying the nutrition of spores, have found that those surviving drastic heat treatment are more exacting in their nutritive requirements than normal spores. The presence of enrichment substances in the media is essential for accurate enumeration, and surviving spores in unsuitable media lose their viability rather quickly. In their studies on bios and related substances Norris & Ruddy(29) and Norris & Hart(30) have found that three separate factors are concerned in the stimulation of respiration, growth and fermentation. The probable constitution of pantothenic acid, one of the constituents of bios, has been given by Williams *et al.*(31).

Werkman and his colleagues have continued their studies on the propionic acid fermentation, and Silverman & Werkman(32) report that vitamin B_1 increases the pyruvate decomposition of propionic acid bacteria. No effect was found with the *coli-aerogenes* bacteria, due presumably to the ability of those organisms to synthesize B_1 (cf. p. 527). This growth factor did not act as co-carboxylase, as aldehyde could not be detected in the propionic acid fermentation. Piret & Fromageot(33), in their metabolic studies on the propionic acid bacteria, conclude that growth factors rather than "complex nitrogen" are essential. These permit the utilization of ammonia nitrogen formed from amino acids; of the ammonium salts studied they found that acetate was the best. Much interest has been attached to the acidic ether-soluble factor essential for the growth of the propionic acid bacteria, and Wood *et al.*(34) found that this, with hydrolysed casein, permitted growth indefinitely; the factor was present in all media giving good growth, and could not be replaced by any known growth factor. Further work has shown(35) that amino acids, although beneficial, are not essential, and that vitamin B_1 and riboflavin can be dispensed with by adaptation.

(*b*) Lactic acid bacteria

General biology

The relationship between temperature and growth is one of the most important aspects of bacteria from the dairying point of view. Claydon (36) has investigated the influence of growth temperature and age on the thermal resistance of *Str. lactis* and found that growth at 10° C. results in higher thermal resistance, thus confirming Anderson and Meanwell's contention that growth at temperatures below the optimum may well result in organisms that would normally be destroyed surviving pasteurization. His results also support Sherman & Hodge in their conclusions that slow growth is correlated with increased viability under unfavourable circumstances. Dorn & Rahn (37) have studied the optimum temperature and related characteristics of *Str. lactis* and *Str. thermophilus* and found the optima to be 34 and 37° C. respectively for growth and 40 and 47° C. for fermentation. The largest number of cells was obtained at 25–30 and 37° C. The rate of fermentation increased when the cells were grown at temperatures above the optimum and the efficiency of fermentation (cell proliferation/lactose fermentation) was greatest at lower temperatures, 2·5–10 times as much sugar being consumed near the maximum temperature. Rahn *et al.* (38) have studied other factors influencing the fermentation of *Str. lactis* and found that *p*H 7 gave the optimum growth, deviations on the alkaline side have less effect than on the acid. The optimum phosphate concentration was 4%, but the highest final acidity was obtained with 6%. The rate was independent of the glucose concentration provided that this did not fall below 0·2%. Lactate and acetoin both retarded the fermentation, but bubbling nitrogen through the culture accelerated it. Variation in age from 2 hr. to 2 days had little effect, and cells could be stored in phosphate buffer at 2° C. for 4 days without loss of activity. Further studies by Rahn & Bigwood (39) have shown that the death of cells held at 0° C. is due to the toxic action of oxygen in the absence of growth mechanisms which normally consume oxygen, as the absence of oxygen results in increased viability.

Tracy (40), in an investigation of the comparative metabolism of *R* and *S* variants of *L. plantarum*, found that the *R* strains produced no acid in 8 days in dextrose broth while the *S* variants produced acid rapidly. The former grew in sugar-free media while the latter did not. It is of interest to compare these results with those of English workers on haemolytic streptococci.

In a study of variations in haemolytic streptococci Dawson *et al.* (41) found three colonial types, mucoid, smooth and rough, in all the Lancefield Groups A to G, except that no mucoid form occurred in G. The occurrence of haemolytic streptococci in the throats, etc., of milk handlers is a matter of general importance, and Foote *et al.* (42) found that of eighty-five such persons examined twenty had β-haemolytic streptococci at least once out of nine occasions and five were persistent carriers. Strains isolated from healthy persons were indistinguishable from those strains of well-known pathogenicity. These investigators concluded that control was impracticable. The streptococci did not withstand drying and died out in the milk, a decrease of about 50% being found during transport under ordinary conditions. Buchbinder *et al.* (43) found that while α-haemolytic streptococci were common in air, β-types were rare. Practically all the latter were of the Lancefield A group. This type was present in 3–7% of normal throats, and the organisms were found to float in the air after evaporation of the droplet. The same workers (44), studying α-haemolytic types, found *Str. salivarius* (Sherman) to constitute 83% of all types. The number in air was a good index of human pollution and varied inversely with the ventilation.

Pullinger & Kemp (45) have found that, although *Str. pyogenes* multiplies readily

at 18–22° C. in sterilized milk, it grows but slowly at 15° C. and only after 48–72 hr. at 18–22° C. in fresh raw milk. Infected cows are considered to be the major source of this organism in milk supplies.

The therapeutic aspects of *L. acidophilus* continue to receive attention, and Weinstein *et al.* (46) were unable to find any correlation between the *p*H and *L. acidophilus* content of the small intestine although in respect of the colon such a correlation was found. Ingestion of lactose reduced the *p*H of the intestine by about 0·5.

Pelczar & Black (47) have studied the "implantability" of *L. acidophilus* in the rat. They failed to implant seven oral strains but successfully implanted intestinal strains, the *R* forms being better than the *S* for this purpose. *L. bulgaricus* could not be implanted.

Identification

A considerable contribution to our knowledge of the relationships of the streptococci has been made by Sherman and his colleagues (48,49,50,51,52,53). These have been summarized in an admirable review by Sherman (54). The streptococci are grouped in four main groups—the "pyogenic", "viridans", "lactic" and "enterococci" on the basis of haemolysis, growth at 10 and 45° C., in 6·5% NaCl, in 0·1% methylene blue milk and at *p*H 9·6, reducing power, resistance to heating for 30 min. at 60° C., and on the ability to form ammonia from peptone. Further tests are used to identify species in these groups. Although some of Sherman's methods have been criticized by other workers, there can be no doubt that the work summarized in this review represents the most important step forward since Orla Jensen's classical work twenty years ago.

Smith (55) has succeeded in isolating *Str. zymogenes* from horse and cow faeces, and Smith & Sherman (56) found that *Str. zymogenes* was by far the commonest haemolytic streptococcus of human faeces, the next most frequent being *Str. durans*, *Str. mastitidis* (*agalactiae*) and Lancefield Group G (?).

Of 313 samples of milk Sherman & Niven (57) found that 8·5% pasteurized and 18% raw contained haemolytic streptococci (not *Str. agalactiae*). Plastridge & Hartsell (58) have studied the biochemical and serological characteristics of streptococci of bovine origin and mention that some strains of *Str. agalactiae* reduce methylene blue in a concentration of 1/5000. They were able to obtain from mastitis-free quarters streptococci which were biochemically but not serologically identical with *Str. agalactiae*. They consider that both serological and biochemical tests are necessary for final identification. Tillett (59) has written a review on the fibrinolytic activity of haemolytic streptococci. Coffey & Foley (60) claim that substitution of asparagine for peptone improves the consistency of the hippurate test, while Bliss *et al.* (61) find that 1 in 10,000 sulphanilamide in broth is bacteriostatic for Lancefield groups A, B and C while having no effect on D and F. Alpha-haemolytic streptococci were also inhibited. Hechler & Farrell (62) have published the results of their biochemical and serological studies on the streptococci, and their results may be compared with those of Sherman (54).

Smit (63) has shown that the "aroma streptococci" may be differentiated from the lactic organisms by the colony appearance on milk agar, and Olson (64) inhibits *Str. lactis* but not the citric fermenting streptococci by α-brom-propionic acid.

Gillespie & Rettger (65) claim that lactobacilli of diverse origin can be differentiated by E_h measurements. Pjetursson (66) finds that *Tbm. lactis* is the most vigorous of the *Thermobacteria*, and produces evidence suggesting that other types may be "derived" from this parent type.

Weiss & Rettger (67) distinguish two types of "*Bact. bifidum*" on the basis of physiological behaviour, and Pederson (68) presents a valuable study on the gas-

producing lactobacilli, provisionally distinguishing five types, *L. brevis*, *L. fermentum*, *L. büchneri*, *L. pastorianus* and a fifth unnamed type (? *Bbm. caucasicum*).

Hassouna & Allen (69) have studied the influence of various factors on the cultural characteristics of streptococci, and found that sterilization of sugars in the medium did not lead to discrepancies in the fermentation tests.

Respiration

Barron & Jacobs (70), in an investigation of oxidation by haemolytic streptococci, found that two distinct types were obtained. One group respired actively and consumed 1·5 mol. oxygen per molecule of glucose oxidized, while the other slowly oxidized sugar with the consumption of only 0·5 mol. oxygen per molecule of glucose. Lactic acid was oxidized directly to acetic acid and carbon dioxide; formic and pyruvic acids could not be detected.

The respiration of the lactobacilli has been studied by Hansen (71) who has shown that Q_{O_2} values are correlated with the main groups *Streptobacterium*, *Thermobacterium* and *Microbacterium*.

The *Thermobacteria* can reduce the reversibly oxidized ascorbic acid to dehydroascorbic acid according to Tkatschenko (72), and a study of the reducing abilities of many types of lactobacilli has been presented by Davis (73) who has shown that certain oxidation-reduction indicators are of value in type differentiation. Rates of growth are correlated with volatile acid production, and it is suggested that enforced slowness of growth in dairy products may be an important factor in flavour production.

Fermentation

Reference has already been made to some biochemical aspects of the lactic acid fermentation. Rudakow (74) has investigated the relationship between lactic acid formation and the presence of granules in lactic acid bacteria, and claims to have detected nucleotides and thymonucleic acid in the granules. These substances are postulated as intermediates. The question of the constancy of the optical form of lactic acid produced has long been a subject of interest, and Katagiri & Kitahara (75) have studied the conditions affecting this matter. While *Leuconostoc mesenterioides* and *Sbm. plantarum* were constant in producing *l*- and *dl*-lactic acid respectively, *L. saké* varied with the conditions. They suggest that this is due to the presence of racemiase. Of other types of organisms tested only *Staphylococcus ureae* produced racemiase. Kopeloff & Kopeloff (76) have similarly studied the optical activity of acid produced by *L. acidophilus* and *L. bulgaricus*. They found that whereas the *S* strains of both produced *d*-lactic acid, the *R* types gave rise to inactive acid. It is evident that the optical activity of the acid is of limited value in identification. Studies on fumarase activity by Kitahara & Katagiri (77) suggest that this may be of use in classification.

Sherman & Stark (78) have adduced evidence that, contrary to views previously held, *Str. thermophilus* hydrolyses disaccharides, and Wright (79) finds that streptococci may hydrolyse and ferment glucosides but that these reactions are inhibited by iodoacetic acid. Davis *et al.* (80) have reported that, of all types of streptococci and lactobacilli investigated under growing and "resting" conditions, only the faecal streptococci (*Str. faecalis* and *Str. liquefaciens*) produced diacetyl. They drew attention to the possible significance of these findings in relation to flavour production in dairy products. Hammer and his colleagues have continued their work on the various factors influencing the production of aroma substances. Agitation with air increased the diacetyl production with both *Str. paracitrovorus* and *Str. diaceti lactis* (81). Studying the effect of *p*H on this fermentation they found that *p*H 3·7–3·9 gave the

highest yield (82). Citric acid was the best of the acidifiers used, lactic acid being poor (opt. *p*H 4·4–4·1) and sulphuric acid moderately good (opt. *p*H 3·6–3·2). The optimum addition of citric acid appeared to be 0·15% (83). Shaking during ripening and holding at 0° C. after the ripening period increased the diacetyl content.

Some progress has been made in the study of the mechanism of the formation of acetoin, and Barritt (84) has found that *Bact. aerogenes* can form acetoin from pyruvate and sometimes from lactic acid, but not from methyl-glyoxal or preformed aldehyde. It was also found that many organisms readily oxidize 2 : 3 butylene glycol to acetoin, and that in the bacterial fermentation of glucose this may precede acetoin. All members of the *coli-aerogenes* group produced acetoin and differences were quantitative rather than qualitative.

Prill & Hammer (85) have described a colorimetric micro-method for the determination of diacetyl, and Michaelian (86) has given a careful account of the sources of error in the normal method of diacetyl determination.

Nutrition and nitrogen metabolism

Most work on the efficiency of media for plating lactic acid bacteria has centred on *L. acidophilus*, and Sabine (87) concludes that liver glucose agar is the best of those tried. Mobley (88) points out that high protein and the presence of milk products in media is associated with the *R* type of colony. Davis (89) has investigated the efficiency of four autolysed yeast preparations and found that while little difference could be observed with the homo-fermentative types, significant differences were detectable with the hetero-fermentative types.

Work on the growth factor requirements has been in progress in a number of American laboratories. Snell *et al.* (90), studying the nutrition of *L. Delbrückii*, concluded that tryptophane was essential in addition to two other factors present in liver, neither being identical with vitamin B_2. Further investigation of one of these factors (91) has shown that it is an acidic ether-soluble substance labile to heat and alkali and apparently different from all other known growth factors. Its effect is evident at a concentration of 0·003 μg. per ml. Experiments with flavins (92) showed that most lactic acid bacteria could dispense with these substances, although for some they were essential. Pantothenic acid was highly active for all lactic acid bacteria (93), and some required nicotinic acid as well. Hutner (94) reports that a number of streptococci require cysteine and another amino acid. Fuller's earth inactivated deproteinized milk.

Werkmann and his colleagues have studied both lactic and propionic acid bacteria (see p. 523) and found that the ether-soluble acidic factor, vitamin B_1 and lactoflavin all stimulated both types (95). Later work (96) showed that *L. büchneri* required tryptophane, and that all three lactobacilli tested required cystine and threonine. Methionine and serine both exerted a stimulating effect. With lactic acid bacteria (97) good growth was obtained with hydrolysed casein, riboflavin, B_1, ether extract of yeast, glucose, ammonium sulphate and salts; cystine stimulated but riboflavin was not essential; although vitamin B_1 was very effective, a few types grew without.

Rahn & Hegarty (98) have found that both ascorbic and nicotinic acids accelerated the lactic acid fermentation, but whereas the former stimulated injured or exhausted cells, the latter exerted an effect on both normal and injured bacteria. It is of interest that according to Balsamelli (99) *L. bulgaricus* and *Str. lactis* can synthesize vitamin C in sterilized milk.

Eagles *et al.* (100) have studied the influence of three factors from tomato, yeast and liver on the growth of *Betacocci* and concluded that they are identical with Bios II A and two components of Bios II B.

Ehrismann & Dramburg(101) report that the deaminating power of streptococci is very weak and that ability to oxidize amino acids is also small. Berger *et al.*(102) has described methods for the preparation of the peptidases of *Leuc. mesenterioides* which are activated by certain cations, especially zinc. They identified two peptidases, three polypeptidases, one acylase but could not detect any carboxypolypeptidase.

Braz & Allen(103) have found marked variation in the proteolytic activity of cultures of streptococci and lactobacilli. There was usually an increase in the protein fraction, apparently at the expense of the proteose-peptone. Streptococci appeared to increase this latter fraction and lactobacilli the amino acids. Chalk increased the proteolysis which could not be correlated with acid production.

Thiel(104) found that appreciable amounts of lactic acid could be produced by growth of lactobacilli in casein digest media free from sugar. This finding is of interest in relation to the growth of lactobacilli in cheese.

(*c*) Starters

Starter troubles continue to be major problems of the cheesemaking industry, and attempts to improve starters sometimes lead to fresh troubles. Davis(105) has discussed the general methods in use for starter propagation and pointed out that cultivation in drastically heated milk may render a starter unfit for use in raw or lightly pasteurized milk. He recommends, therefore, an "acclimatization stage" for the starter in milk heated momentarily to 200–210° F. Rice(106) has studied many factors influencing starter growth and found that *Str. lactis* and *Str. cremoris* were about equal in activity. After subculturing at 2-day intervals for 6 months cultures were mostly *Str. lactis*. Milk heated to 180° F. or for 30 min. at 160° F. was superior to raw milk as a medium; further heating had no effect. The shape of the containing vessel and stirring the culture were apparently without effect. He found further that cooling the culture after clotting had no effect except that prolonged cooling weakened the starter. Experiments with various type cultures showed that *Bact. coli*, *Bact. aerogenes* and a *Sarcina* had no effect, *B. subtilis* stimulated, and that inhibiting streptococci were effective if previously grown in the milk but not if simultaneously inoculated. The effect of mastitis milk was irregular, most samples depressing but some stimulating growth. The effect was still evident when only 10% mastitis milk was present. Growth in late-lactation milk was appreciably slower. Aeration decreased the rate of growth, and bubbling nitrogen caused an initial retardation possibly due to loss of CO_2. Colostrum stimulated acid formation.

Elliker & Frazier(107) have found that the time and temperature of incubation of cultures of *L. helveticus* and *Str. thermophilus* are of great importance in determining the activity. Similarly, Nusbaum & Price(108) have shown that these factors will control the proportion of *Str. thermophilus* and *L. bulgaricus* in high-temperature starters. Elliker & Frazier(109) report that fresh is superior to stale milk, and that steaming is not so effective as autoclaving.

Langhus *et al.*(110) failed to correlate quality of Brick cheese at 7–8 days with milk quality (methylene blue test). Troubles were traced to undesirable types of organisms in the whey starters used.

"Slow starter" is without doubt the most common fault in cheesemaking, and Whitehead and his colleagues have continued their investigation in New Zealand where *phage* appears to be the major cause of the trouble. Their earlier work was concentrated on finding an empirical solution to the problem, and they found that the use of heavy inoculation and an aseptic technique in starter propagation was most effective(111). The relation of "abnormal milk" to "slowness" could not be confirmed.

They had found that the following factors favour phage development—aeration of milk before inoculation, ageing of the milk and the use of light inocula. All these factors increased the lag phase, and they now recommend (112) 1–1·6% inoculum into freshly heated milk and immediate incubation at 24° C. for 24 hr. Phage could frequently be isolated from the whey of normally working vats in the later stages of the making process. Nelson (113) has shown that under certain conditions strains of *Str. lactis* may be sensitive to filtrates from slow cultures.

O'Droma & Grimes (114) have isolated *Bact. coli communior* from a dried starter preparation, a fact which emphasizes the need for continual bacteriological control in this work.

The aroma of butter and the factors controlling it have received considerable attention, and Davies (115) has given a useful summary of the subject. Wilster (116) found that starter invariably increased the score and that the best results were obtained when the cream was ripened and then held cold. Hammer & Slatter (117) have studied the factors influencing the changes in aroma substances. The highest yield was obtained at high temperatures and low *p*H values, but was usually increased by a larger addition of starter and sometimes by adding cultures of citric acid-fermenting streptococci. The disappearance of the flavour substances was presumably due to their reduction to 2 : 3 butylene glycol by the streptococci. Hammer (118) found that mixtures of *Str. diaceti-lactis*, *Str. citrophilus* and other streptococci were not so good as *Str. lactis* with *Str. paracitrovorus* and *Str. citrovorus*.

(*d*) Cheese

General

A second edition of a book on soft and cream cheese by Rosell (119) has appeared, and investigations of alcohol-glycerol rennet by Davis (120) and of annatto by Carrie (121) have been published. Nicol (122) has written a useful summary of the scanty knowledge available on the microbiology of salt.

An important aspect of cheese development is the packing of cheese in containers or pinwrapgs. Investigations on the canning of Cheddar cheese have been reported in America (123) and in England (124). Goss (125) has found that ripening small cheese in liquid paraffin leads to more rapid ripening, a characteristic softness and very little loss in weight. McCubbin & Reichart (126) found that the use of paraffin and cellophane with paraffin led to the development of unclean flavours. Mould was prevalent on the cheese covered with cellophane. A temperature of 40° F. was invariably superior to one of 70° F. for ripening, the latter leading to gassiness and fermented flavours.

In New Zealand considerable attention has been paid to tests for assessing the quality of milk. Comparisons of the curd, reduction and microscopic tests showed 93–95% agreement. It was concluded that immediate cooling was a factor of prime importance (127). Stocker (128) has drawn attention to the effect of copper on the fermentation test. Dissolved copper inhibits lactic and propionic acid bacteria but not coliform organisms. Rogers *et al.* (129) have shown that the grading of cheese is correlated with the quality of the milk and the *p*H at certain stages. According to Marquardt (130) poor quality in winter is a major problem which cannot be explained by temperature differences. Increasing the calcium increased the rate of mellowing. Hass (131) has investigated the effect of colostrum on cheesemaking. It retarded rennet coagulation, giving a soft curd with expression of whey. Hussmann (132) reports that morning is inferior to evening milk for starter growth, and they react differently in reduction, catalase and fermentation tests.

The vexed question of mastitis in relation to cheesemaking has been investigated

by Davis & McClemont (133). Of infected milks 38%, and of normal milks only 8%, reacted slowly with rennet. A new test—the brom-cresol-purple rennet test—for testing milk for cheesemaking is described. About half the infected milks gave slow growth of starter and cheese made from infected milk of *altered chemical composition* was of weak body. Similar results have been reported by Nyiredy (134). Davis (135) has also shown experimentally that mastitis milk is likely to result in off flavours and poor texture in the cheese. Marquardt & Hucker (136) have made satisfactory cheese from infected milk by adding hydrochloric acid or calcium chloride.

Investigations have also been published on methods of treatment of milk for cheesemaking, especially with a view to the removal of adverse factors such as excess cells due to mastitis. Matheson *et al.* (137) state that clarification results in a higher oxygen content but that according to *p*H measurements the efficiency of starter cultures is increased. Cheese from unclarified milk contained more water and was softer in texture, and they conclude that the process greatly improves mastitis milk for cheesemaking. Dorner & Staehli (138) also found that clarification reduced the tendency to faults and doubled the number of first-grade cheese.

The use of silage milk for cheesemaking has received further attention and Rodenkirchen (139) has found that the use of silage with a high content of butyric acid bacteria results in cracked cheese. Feeding such silage greatly increased the numbers of butyric acid bacteria in the dung of the animals. Funder (140) concludes that A.I.V. silage is usually safe to use when Gouda cheese is made, but there is a liability to a butyric fermentation if the starter is weak.

Much interest has lately been taken in the effect of pasteurization on milk for cheesemaking. Marquardt (141) states that for semi-soft cheese good raw milk is better than good pasteurized and suggests that enzymes in milk play a significant role in such cheese. Orla Jensen (142) has reviewed the position and points out that pasteurization will destroy coliform but not butyric acid bacteria; for the suppression of the latter saltpetre is the only method available. He gives interesting figures showing the behaviour of different types of lactic acid bacteria in milk heated to various degrees. Tuckey (143), Clausen (144) and Taylor (145) have made practical contributions to the problem, while Lembke & Tolsdorf (146) found that flash pasteurization had no effect on the flora or *p*H of Dutch cheese and has given useful data of the manufacturing processes of these cheese. A review of the literature on pasteurization of milk for the manufacture of continental varieties has been published (147), and Davis (148) has summarized the advantages and disadvantages of the practice, pointing out that the flavour problem in cheese made from pasteurized milk can be partly solved by the addition of selected strains of *L. casei* or the use of a small quantity of good quality raw milk (see also p. 531).

Scientific contributions to the manufacture of Swiss cheese have been made by Rogers (149), Götel (150) and Goss *et al.* (151), while Dorner *et al.* (152) and Kursteiner (153) have investigated the methods of controlling the number of propionic acid bacteria by the use of added cultures. According to Hostettler (154) there is no simple relationship between the residual lactic acid and the eyes in Emmenthal cheese. He claims that toughness could be combated by the use of pure cultures of propionic acid bacteria. Attempts to suppress faults in Tilsit cheese have been described by Schwarz & Beinert (155), and Dibbern (156) has reported on the use of the Nilson cheese machine in Sweden. McDowall (157) has investigated the effect of temperature on storage of cheese. No deterioration was found at 31° F. and the quality was slightly better than at 45–50° F. At 14° F. the body deteriorated badly and the cheese developed a soapy, tallowy flavour.

The importance of limited *p*H ranges for quality in Cheddar cheese has already been emphasized by American workers, and Dorner (158) reports similar results for

young Emmenthal cheese. He found that 88% of first-grade cheese had a *p*H value of 5·11–5·30 at 24 hr. Cheese whose *p*H values were 5·06 and 5·35 were invariably of bad quality.

Pyne & O'Droma (159) have reported interesting experiments on the role of albumen in cheesemaking. Their results are in opposition to those of Samuelson and are of importance from the point of view of the use of pasteurized milk.

Ripening (including bacteriology)

Freeman & Dahle (160) have studied the influence of a number of factors on the ripening of cheese and have found that the rate of proteolysis is proportional to the number of bacteria initially present in the cheese, although there was apparently no correlation between the latter and the development of flavour. Contrary to the findings of most other workers they report that cheese ripened at 63° F. attained as high a flavour score as those ripened at 45° F., although bitterness was favoured by high temperature. The addition of pure rennin increased the proteolysis and slightly accelerated flavour development; pepsin increased proteolysis but not flavour and trypsin greatly increased proteolysis and flavour production but adversely affected the final score. Bogdanov (161) has found that a peptonizing *Str. lactis* (Gorini's mammococcus) accelerated ripening but tended to produce bitterness. Davis *et al.* (162) have studied the degradation of milk proteins by various combinations of lactic acid bacteria, rennet enzymes and rennet micro-organisms, and conclude that the first two can account for the non-protein nitrogen in Cheddar cheese; the relatively low amino-nitrogen figures obtained are attributed to the relatively more acid conditions in the cultures studied.

Attention has been paid to bacteriological factors responsible for flavour development. Babel & Hammer (163) report that some of the effects of adding propionic acid cultures to Swiss type cheese can be simulated by adding calcium propionate. Sherwood (164) has isolated lactobacilli responsible for desirable and undesirable flavours in Cheddar cheese and reproduced these characteristics by inoculation of the specific organisms into the cheese milk. Similarly, Orla Jensen (165) claims that by using a culture of *L. helveticus* small cheese of the Gouda type develop the typical Emmenthal flavour. Hansen (166) has also investigated the effect of certain types of lactobacilli on the ripening of cheese.

Lane & Hammer (167) have found that some types of *L. casei* and of *Propionibacterium* produce desirable flavours in cheese. The acid number of the fat increased during ripening and was higher in cheese made from raw milk than in that made from pasteurized. The volatile acids were likewise higher in the former.

The study of the numbers of bacteria in cheese at various stages has thrown some light on the reactions involved in ripening. Owing to irregular distribution and colony formation as well as to the difficult nature of the material, the enumeration of organisms in cheese may be very misleading.

Harrison (168) has shown that emulsification of cheese in making bacteriological counts results in figures from two to ten times as high as those obtained with the ordinary grinding technique. He also observed that counts varied widely from place to place in the same cheese. Changes in count during ripening have also been studied by Schmiege & Frazier (169), who found that *Str. lactis* and *Str. thermophilus* in Brick cheese died out and were replaced by those types present in Swiss cheese. Carey & Frazier (170) have, in addition, found that *Str. thermophilus* gave a slower but steadier development of acidity than *Str. lactis* when these were used as starters for Brick cheese. Meyer (171) has described a comprehensive investigation of the bacteriology of Tilsit cheese. The streptococci disappeared in 2 months and the lactobacilli were at

a maximum at from 1½–2½ months, while the numbers of the acid- and rennet-producing cocci remained more or less constant. Coliform organisms had usually disappeared in 3 weeks. The use of poor quality milk resulted in appreciable differences, there being fewer lactic acid bacteria and more putrefactive and spore-forming organisms.

Kelly (172) has studied the surface microflora of Limburger cheese and found that in the best cheese the yeasts which predominated in the early stages (1–5 days) were replaced by *Bact. linens* (6–18 days). Drewes (173) has similarly investigated the types of micro-organisms in sour milk cheese.

Faults

Price & Spicer (174) have studied the relation between acid defects and *p*H in Brick cheese and state that if the minimum *p*H is 5·15 (3–5 days) the cheese will not be excessively acid. The fault-producing potentialities of coliform organisms in cheese are still fruitful sources of trouble in cheesemaking. According to Varga (175) a high content of coliform organisms in Trappist cheese may be due to infected udders. A similar finding has been reported by Stocker (176). Florentin (177) has investigated the numbers of coliform organisms in various types of cheese. High-scalded cheese such as Gruyère were free from them; less highly scalded cheese such as Dutch and Port Salut contained a few, and the soft varieties such as Pont l'Evêque contained more than 1000 per g. In the last case coliform organisms are considered to contribute to the flavour.

Schwarz *et al.* (178) have studied the effect of various substances on the blowing of cheese by the *coli-aerogenes* group of bacteria which, as is well known, is inhibited by saltpetre. The factors influencing the amounts of hydrogen and carbon dioxide produced were also investigated.

Hanson *et al.* (179) have described a characteristic splitting of Brick cheese due presumably to a spore-forming anaerobe. It was more prevalent in sweet cheese and moisture was apparently not a controlling factor. "Sterilization" and pasteurization did not obviate the defect, but some control could be obtained by the use of high acidities, high salting and the making of small cheese. Ritter (180) suggests that infection with *Cl. butyricum* may be due not only to the use of silage milk but also to poor factory technique.

Kieferle & von Jahn (181) have made the interesting observation that if hydrogen is present in the gases of cheese, the latter arise through a "fault fermentation". Nevertheless, such gases may contain some hydrogen without necessarily reducing the quality of the cheese.

Dorner & Thöni (182) have shown that the foul-smelling secondary fermentation or grey rot of Emmenthal cheese is due to *Bact. proteolyticum*. The fault is accompanied by a marked rise in *p*H, proteolysis and a grey discoloration, and occurs after 4–5 months.

Koestler (183) has described the origin and characteristics of "bi-coloured" curd in Emmenthal cheese, in which slow eye formation, "bi-coloured" curd and brown spots frequently occur together. He suggests that the fault is due to conditions in the fresh cheese arising from irregularities in fermentation.

Breed & Peterson (184) found that "rusty spots" in Cheddar cheese were due to pigment-forming variants of *L. plantarum* and *L. brevis*. Factors influencing "saltpetre colouring" in cheese have been studied by Knudsen *et al.* (185), who found that the salt content was without effect and that the fault only occurred when the *p*H was below 6.

According to Valentine (186) there are two causes of mould penetration in New Zealand cheese, detachment of the crown or bottom and small fractures or cracks.

Blue-veined cheese

Blue-veined cheese are now attracting considerable attention, especially in America. There is a demand for Roquefort cheese there, and the efforts of scientists and practical cheesemakers have been aimed at perfecting methods for the manufacture of cheese of this type from cows' milk. Reports of the successful manufacture of "Blue" cheese in America and Canada are given by Lane (187), Macy (188) and Irvine (189), in which the methods of manufacture adopted at various centres are discussed.

Since the legislation of 1925 reserves the name Roquefort for cheese made wholly from sheep's milk, strict control is necessary in order to avoid the marketing of imitations under this name. This point has been emphasized by Génin (190), who referred to the need for a method which would quickly and with certainty determine whether a cheese is a true Roquefort, and in this connexion reviewed the work of Garard (191). By the use of a carefully standardized method for determining Polenske numbers, Roquefort cheese can be distinguished from its imitation.

The normal period of ripening of a "Blue" cheese (Roquefort type) is not less than 6 months. Ways and means of shortening this period and yet offering a satisfactory cheese have been sought. Lane & Hammer (192, 193, 194) found that the use of homogenized raw milk, though necessitating modification in the manufacture, would shorten the ripening period by about 2 months. A definite rancidity developed during the early stages of ripening, but this later blended with the volatile acids yielding a more desirable flavour. The use of homogenized pasteurized milk would control certain defects, but the ripening period would be longer than when homogenized raw milk was used. The addition of a lipolytic enzyme has been tried by Combs & Coulter (195). The hydrolysis of the fat was hastened to some extent, and the cheese had a "peppery" flavour in $4\frac{1}{2}$–5 months. The flavour was not typical but was acceptable to many.

Golding (196, 197) has studied the problem from the point of view of supplying the mould with the atmosphere most favourable for growth. He suggested that the presence of CO_2 rather than O_2 was the limiting factor in determining the growth of mould within the various types of blue-veined cheese. Pricking the cheese allowed the circulation of air, and this could be hastened by subjecting the cheese to alternating reduced and normal pressures. The practical application of these findings presents many difficulties.

A detailed study of the taxonomy of the fungi of blue-veined cheese (twenty cheese of eight varieties) was made by Dattilo-Rubbo (198). He found that *P. roqueforti* Thom. was the dominant type, and that this could be subdivided on colony characters into three classes, each characteristic of certain types of cheese. Bryant (199) has demonstrated that eight strains of *P. roqueforti* each had the power of causing the destruction of various fatty acids in culture media. This was regarded as support for early suggestions that the moulds use the volatile fatty acids in cheese as food for growth. Caproic, caprylic and capric acids were toxic in 0·1 % concentrations but not in 0·05 %. By the action of the mould on caprylic acid, methyl-*n*-amyl ketone, an important flavour constituent of Blue cheese was formed (200). It is probable that this reaction normally occurs during the early stages of the ripening of the cheese when the mould is actively growing. Later, when the mould activity abates, caprylic acid accumulates.

A useful résumé of recent work on factors affecting the ripening of Blue (Roquefort type) cheese has been given by Lane & Hammer (201).

Determination of the volatile acid content is frequently used to assess the degree of ripeness of blue-veined cheese. Steam distillation of a cheese mush is the method usually adopted to obtain this figure. Hiscox & Harrison (202) have pointed out

probable inaccuracies in this figure resulting from the retentive power of various cheese constituents. To avoid these inaccuracies they (203) have suggested a method in which the acids are extracted from the cheese components before steam distillation of the solutions. The figures obtained for Cheddar cheese were twice as great and for Stilton three to four times as great as those obtained by simple steam distillation.

(*e*) Butter

The great problem of the butter industry is to provide butter of good quality which will suffer no serious deterioration during cold storage, or when subjected to the conditions imposed by exposure for sale and storage in the consumer's house. This problem is not merely a question of bacteriology. Chemical aspects as well as methods of manufacture are involved, and none of these factors can be considered as a separate entity.

The grade of butter at the creameries is not necessarily a good criterion of the quality of the butter that will eventually reach the consumer; the keeping quality of the butter under storage conditions should also be determined. To this end it is necessary to re-score the butter after suitable storage and to note the degree of deterioration during the period. Jacobsen (204) used storage for 7 days at 21° C. Parsons (205) has advocated storage for 14 days at 15·5° C. as giving more reliable results than 7 days at 20–21° C. Flake & Parfitt (206) used storage for 10 days at 15·5° C. This lack of uniformity in method, and therefore in the interpretation of results, is apparent in all the branches of study but is inevitable when so many problems are under examination and when individual points of view must be so varied. The sum total of the findings can therefore offer only a partial solution of the problems and must be mainly indicative of the lines along which further research is urgently required.

The presence of yeasts and moulds in butter has been regarded as one of the most important factors in deterioration in quality. The determination of the yeast and mould count has thus become an integral part of the bacteriological analysis of butter, but it is probable that the findings have frequently been given an interpretation that was misleading. Macy (207) has produced evidence that, although there is a tendency for butter with a low yeast and mould count to withstand storage better than samples with high counts, the keeping quality of a sample cannot be confidently predicted from the yeast and mould count. A low count, however, may be taken to indicate the use of a high-grade cream and careful methods of manufacture, and the chief value of the count lies in this direction. The comparative freedom from mould of well-produced butter was noted by Block (208). The main sources of yeasts and moulds have been discussed by Macy (207), Cordes (209) and Vernon (210), and the general conclusion was that the seat of effective control is the factory. Parfitt (211) has even gone so far as to suggest a standard for effective pasteurization of cream and efficient manufacture of butter based on the yeast and mould count of the finished product. Brown & Parfitt (212) have suggested that the phosphatase test, using the serum obtained by centrifuging the melted butter, might serve as a criterion of efficient pasteurization of the cream. As they point out, however, there is a possibility that the manufacturing process of the butter might influence the phosphatase reaction.

Counts of the lipolytic, proteolytic and total bacteria in butter are usually included in the bacteriological analysis. In this connexion, Flake & Parfitt (206) found a high correlation between proteolytic counts on tryptone-skim milk agar and keeping quality and between lipolytic counts on tributyrin agar and keeping quality. On the other hand, Major (213) found that a high proportion of commercial butter samples tested by him on Nile blue-sulphate-beef infusion agar showed the presence of lipolytic organisms, but none of the samples developed rancidity during the keeping

quality test. Also Jacobsen (204) found large numbers of proteolytic and lipolytic organisms in certain samples of unsalted butter held at room temperature (21° C.) for 7 days, but there was no significant correlation between these numbers and the development of "off" flavours.

In the present state of uncertainty as to the significance of the bacteriological analysis of samples of butter it is not surprising that the methods of examination should vary according to the point of view of the worker. Direct microscopical examination of butter has proved to be of value. Certain refinements in the technique of the preparation of the serum before proceeding to the Breed count have been introduced by Demeter (214). Hussong (215) has described a method, not intended for counts, which gave a good picture of the bacterial flora of butter. The presence of large numbers of rod-shaped organisms was found by Flake & Parfitt (206) to be associated with low keeping quality. Hedrick (216) found that the number of organisms found microscopically in butter serum was a fair index of quality when the butter was made from high-grade cream, but the test was not reliable for butter made from low grades. In view of the importance of the yeast and mould count special studies have been made of media suitable for this purpose. Parfitt (217), Parker (218) and Shadwick (219) each appreciated the merits of potato dextrose agar, and Parfitt proposed a dilution method by means of which irregularities in the 1 ml. counts, due to the difficulty of obtaining good distribution of fat, would be obviated. Parker found this method a distinct improvement. The Burri Smear technique has been used by Long & Hammer (220) in the examination of butter. In this way they demonstrated marked irregularities in the distribution of the bacteria, thus emphasizing that the growth of bacteria in butter is largely limited to certain areas, presumably infected water droplets. The variations in composition (moisture and salt) in different portions of butter from the same churning, as determined by Hood & White (221), were thought to be unjustifiably great. They suggested increased control of the manufacture of butter so that greater uniformity of composition might be secured.

Not only the grade of cream used but also the degree of the ripening allowed affects the keeping quality of butter in storage. Holm *et al.* (222) found that samples of butter made from cream of acidity of 0·13–0·19% deteriorated at practically identical rates, and that butters made from cream with acidity of 0·3% had lower keeping quality than those made from sweet cream. Martin *et al.* (223) have also reported that butter made from high-acid cream deteriorated rapidly on storage—the best butter was made from fresh low-acid cream. The difficulties of producing butter which will not seriously deteriorate in storage are accentuated where there is a demand for unsalted butter. The influence of the acidity of the cream in these circumstances has been studied by Wiley (224), who found that the acid in butter stored at 65 and 40° F. had little power to delay deterioration due to bacteria. At 0 and 14° F. the greatest deterioration occurred in butter made from cream at *p*H 5. There was less deterioration in butters which were acidified to the same degree by the addition of pure lactic acid. A similar finding has been reported by Boogaert *et al.* (225) who recommend, for the preparation of good butter which will withstand cold storage, the use of unsoured cream and the addition of 0·5% HCl to the last wash water. In the absence of the inhibiting action of salt various yeasts, moulds and bacteria are able to develop in unsalted butter and may have a deleterious effect on the quality. Storage at very low temperatures (e.g. −25° C. as reported by Jacobsen (204)) causes a greater decrease in the bacterial count in unsalted butter than in salted, but in the latter the salt is the more effective destructive agent.

The popular preference for a full-flavoured butter is referred to by Pont (226) as justification for the careful consideration of improving the flavour of sweet cream butter by the addition of diacetyl. The dangers of such a procedure are well known,

hence Parker & Shadwick (227) favoured the addition of good starter distillates which would provide the desirable flavours but avoid the addition of undesirable bacteria. This method had already been suggested by Ruehe & Ramsay. The chemical control could be more exact than the bacteriological.

"Off" flavours in butter, due to micro-organisms, are all too frequent, surface taint being one of the most common. Hammer (228) has isolated *Achromobacterium putrefaciens* from many samples of defective butter. This organism could not be found by ordinary plating, but was obtained by smearing small portions of butter directly on the surface of agar plates. The "rabbito" taint was the subject of a long investigation by Cullity & Griffin (229). Their results were not conclusive but suggested that the trouble was of bacterial origin, and was probably water-borne. A musty flavour was traced by Randell (230) to an undescribed species of *Achromobacterium*. This also appeared to be water-borne. In view of the danger of infection of butter by the wash water, Olson's (231) findings are of interest. The use of infected water did not materially affect the bacterial content of the freshly made butter, but it might seriously increase the amount of deterioration on storage. A Seitz filter removed water-borne organisms. In general, moulds are responsible for a considerable amount of deterioration in butter, but the specific action of the individuals has not received much attention. Vernon (210) has described moulds concerned in the internal moulding of the butter, and Ause & Macy (232) have studied the influence of seven strains of *Oospora lactis* on the keeping quality of butter. They found no direct correlation between the mould count and the quality of the butter after storage, and concluded that this mould was not a factor in deterioration of butter. Nevertheless, the addition of salt improved the keeping quality. In view of the frequent occurrence of this mould in all dairy products the detailed study of sixty-one cultures from Canadian and American butter, a preliminary report of which has been given by Macy & Gibson (233), is of particular interest. The mould is sensitive to salt—according to Kanunnikowa (234) 1 % sodium chloride is partially inhibitory to its growth. Neither is its resistance to heat in any way remarkable. Tanner & Hofer (235) showed that of 224 cultures isolated from cream from a wide area, none was able to resist pasteurization in cream at 62·8° C. (145° F.) for 30 min. One fairly resistant strain was able to resist 20 min. at 145° F. in phosphate solution. It seems that, as yet, the significance of this common mould is by no means established.

IV. PASTEURIZATION AND OTHER PROCESSES

(*a*) Pasteurization

The papers on pasteurization which have come to our notice since the last review show that the viewpoint is changing, and interest is rapidly passing from purely scientific and experimental to practical and industrial problems. Few of the papers add to our scientific knowledge, they are mainly concerned with industrial successes and failures or with the advocacy of pasteurization in general. There are numerous descriptions of pasteurizing plants in various dairies, with mention of points of management and equipment that are of special interest. Gear (1) has described a modern form of batch pasteurizer designed for the smaller dairyman. A mechanical circulation of the heating medium controls the temperature of the milk.

Interest in the high-temperature short-time process is maintained in spite of the very limited official recognition it has received. In America this method has been permitted in several states for some years, and recently it has been introduced into Chicago to a limited extent for the purpose of observation and trial under commercial conditions. Krueger (2) has reported on this trial which appears to have been eminently

satisfactory. Heating at 161° F. for 16 sec. is suggested as a suitable temperature/time standard. New types of plant and improved forms of old have been under test. Reports of tests on four such machines have been issued by the Danish Dairy Research Station. The *Stassano* machine (3) has been greatly improved in design since its first introduction. The most marked change is in the length of the heating tube which has gradually been reduced from 14 to 2 m. The time of heating has, in consequence, fallen from 15 to 1·4 sec. This type of machine in which the milk was heated to 73–74° C. in 1·4 sec. was found to be satisfactory for the destruction of tubercle bacilli, coliform organisms and general bacterial flora. The keeping quality of the milk was improved and the general chemical composition not significantly changed. The phosphatase test (Stein's method) was found, however, to be positive under these conditions. The inclusion of a holder in which the heating period was prolonged by 5·4 sec. was necessary to give a negative phosphatase test. The *Spirala* (4) was a vertically erected plate machine in which the milk flowed through spiral canals on the surface of the plate. An unusual feature of this machine was the fact that the milk was held at 69° C. for 21 sec. before being raised to the pasteurizing temperature, 72° C. (161·6° F.), in 6 sec. There was satisfactory destruction of tubercle bacilli at 70–73° C., of *Bact. coli* at 69–74° C. and of the general bacterial flora at 72° C. The chemical properties of the milk were not changed. The A.P.V. machine (5) consisted of the usual type of stainless steel plates and included a holding section in which the milk was retarded for 23 sec. Tubercle bacilli in naturally infected milk were destroyed at temperatures from 69·5 to 72° C. Coliform organisms were not found in milk pasteurized at 68, 69 and 70° C. No significant change in the chemical composition of the milk was noted below 72° C. The fourth type of machine, the "Kolding" Plate Pasteurizer, incorporated a holding section in which the milk was held for 31·5 sec. Tubercle bacilli in naturally infected milk were killed when the milk was heated at 69·5–70·5 and 70·5–71·5° C., and coliform organisms were destroyed at 68° C.

The advantages of the H.T.S.T. method were pointed out by Farrall (7), who mentioned a modification in which the milk is raised to the pasteurizing temperature, partially cooled and again raised to the pasteurizing temperature before the final cooling. This method should improve the efficiency of destruction of bacteria, but few trials of this process have been published. Mattick & Hiscox (8) have described experiments on commercial plants which demonstrate the efficiency of heating at 162° F. with 15 sec. holding in the destruction of bacteria, especially pathogenic and thermophilic organisms.

Numerous papers are concerned with various aspects of the control of the efficiency of pasteurizing plants and many deal with the phosphatase test which has proved to be of the greatest value for this purpose, but a detailed review of these cannot be made here. Mechanical tests of plant, as was pointed out by Tiedeman & Swanner (9), are important and have led to improvements in design. Bacteriological testing cannot, however, be omitted, and the usual standard of comparison has been "laboratory pasteurization", i.e. the heating of a representative sample of the raw milk in the laboratory under controlled, identical conditions of time and temperature. For high-temperature short-time pasteurization these conditions are not easy to imitate, but a "standard heater" of the type described by Plock & Wälzholz (10) should be very useful for this purpose.

It is generally recognized that a special medium is required for plate counts of pasteurized milk. A nutrient medium containing separated milk is widely used. Guittonneau *et al.* (11) used a tryptic digest of skim milk as the basis of a medium which they found compared favourably with the tryptone medium of Bowers & Hucker and the lactose medium of Demeter. Guittonneau (12) used this medium in the study of the

efficiency of a pasteurizing plant. The usefulness of the microscopic counts for pasteurized milk has been reiterated by Ward & Myers (13), who maintain that the numbers of dead bacteria which take the stain and so remain visible after pasteurization are insufficient to affect the value of this test. The importance of the "coliform" test is often questioned because of the existence of heat-resistant strains of coliform organisms. According to Lembke (14) this resistance is not a constant property, but merely a result of the physiological state of the organism. A significant decrease in the resistance of coliform organisms inoculated into milk may occur during the period necessary for testing the plant. For this reason he recommended the use of a "standard heater" such as that mentioned above, rather than the coliform test for plant control.

The deleterious effects of numbers of thermophilic organisms in commercial pasteurized milk are well known. One case in which the milk developed an unpleasant taste and smell was described by Franco (15), and another was referred to by Mattick & Hiscox (16). In the latter case the trouble abated during a temporary substitution of high-temperature short-time pasteurization for the holder pasteurization, using the same milk supply. The presence of thermophilic organisms may often be demonstrated even in efficiently pasteurized milk, and they are usually present in large numbers where the methods of pasteurization are open to criticism. Carefully produced raw milk should contain few thermophils. Thomas (17) found that 78% of 203 samples tested were free from thermophils, and with few exceptions only those produced under unsatisfactory conditions were infected. Groppali (18) has identified and compared the types isolated from raw and pasteurized milk. All were spore-bearing bacteria.

The advantages of pasteurization of milk for cheesemaking have long been recognized. They have been discussed at some length by Price (19), who has also reviewed the literature relating to the manufacture and ripening of cheese made from pasteurized milk. Tuckey (20) also has advocated this procedure on the grounds of the greater uniformity of quality of the cheese and the protection from pathogenic organisms. Both holder and high-temperature pasteurization have been used for this purpose, and Price & Germaine (21) have described an inexpensive type of plant that can be adapted to either method. Experiments have been carried out at the National Institute for Research in Dairying (22), to study the use in cheesemaking of milk pasteurized, at temperatures varying from 155 to 180° F., by the high-temperature short-time process. The cheese made from the pasteurized milk developed more slowly than those made from the corresponding raw milk, and were still unmistakably immature when the latter had acquired a full flavour. Demeter (23) has found that certain bacteria of importance in dairying are sensitive to the "agent" in raw milk which lowers the heat resistance of coliform organisms. This is important in cheesemaking, since useful bacteria may be destroyed or, on the other hand, harmful organisms may escape. This latter is more probable with "high pasteurization" than with the holder process, since the agent itself seems to be destroyed at these temperatures, and even if not destroyed a definite interval of time appears to be needed for its action (see also p. 530).

(*b*) Other processes

The continuation of large-scale trials of the Hofius process for the preservation of liquid milk over long periods has led in all cases to similar results. Raw milk kept at a temperature not higher than 10° C. (50° F.) under an oxygen pressure of 8–10 atm. was reported to remain "good" for periods varying from 4 to 8 weeks. The milk soon acquired a flat taste and the flavour and odour gradually deteriorated, but souring did not occur. At first the bacterial count fell but then gradually rose again. This is

explained by the destruction, under the high oxygen pressure and low temperature, of the general flora of the milk, such as the lactic acid bacteria and coliform organisms, accompanied by the survival and multiplication of resistant weak acid-producing cocci. In some trials, rod forms predominated in pasteurized milk which had been stored under these conditions. Details of the various experiments are to be found in papers by Rodenkirchen (24), Mohr *et al.* (25), Schwarz (26), and van Beynum & Pette (27). The Hofius-Richter modification (28) by which the milk is heated at 55° C. under an oxygen pressure of 10 atm. for 5 hr. immediately after being placed in the storage tank causes a greater destruction of bacteria, and the milk has a better keeping quality. Van Raalte (29) reported that the milk kept for at least 6 weeks, and that very few living bacteria remained. Vitringa (30,31) also found that milk treated by this process would keep for at least 6 weeks, and that pathogenic organisms, including tubercle bacilli, were killed during the heat treatment. If it was not desired to keep milk for more than 1 week the preliminary heat treatment could be reduced to 1 hr.; less than that would not destroy tubercle bacilli. Both these investigators are of the opinion that this process is worth consideration as an alternative to pasteurization.

Very little new information is available on the application of physical agents other than heat to the sterilization of dairy products. A review of the present position has been given by Harry (32). A new type of ultra-violet lamp, described by Porter (33), appears to offer great possibilities in this and other fields. A great deal of work on the bactericidal action of ultra-violet rays is being carried out by French scientists in particular, but the results are not yet such as to permit industrial application.

(*c*) Canned and dried milk products

Modern conditions necessitate the preparation of foods for storage and raise special problems in the milk industry. A general review in *Canned Foods and the Canning Industry*, one section of which is devoted to milk products, has been given by Woodcock & Lewis (34). Spoilage due to purely chemical factors may cause serious losses, but bacterial faults are also of frequent occurrence. The organisms responsible are usually spore-bearing bacteria, which alone are able to survive the temperature at which the product is processed. Nichols *et al.* (35) showed that bitterness and thinning in canned cream was due to strains of *B. subtilis*, the spores of which would withstand a temperature of 120° C. for as long as 40 min. Not all strains of *B. subtilis* were able to produce this fault. Coagulation in tins of evaporated milk has frequently been traced to the presence of *B. coagulans*. This organism has been studied in detail by Sarles (36) with special reference to its heat resistance. Of nine strains tested, six readily produced spores, especially on agar media, and in general these strains were more resistant than the other three which sporulated sparsely.[1] The obvious methods of control are selection of good milk supplies and careful plant management. It is rarely feasible to apply sufficient heat treatment to kill spores that have gained access to the tins.

With the increasing use of milk powders in the preparation of various foods, the purity of the powder is of the greatest importance. The ideal condition is not always attained. Crossley (37) found that powder produced by the spray process from a good milk supply had a lower bacterial content than that made from an inferior supply, but the types of organisms were similar. Yeasts were rarely found, but a few moulds were sometimes present. Acid-forming species nearly always predominated, thermoduric cocci forming the bulk of the flora. Aerobic spore-formers were always present, usually only in small numbers, and anaerobic spore-bearers and coliform organisms

[1] Other references to heat-resistant organisms are to be found in the section devoted to spore-bearing bacteria.

were occasionally detected. The numbers of bacteria decreased during storage but not at a constant rate. Much seemed to depend on the original flora. Very similar findings have been recorded by Halverson & Mleynek (38). The detection of thermophils in skim-milk powder is important because of its increasing use in the preparation of tinned soups and similar foods. Sorensen (39) has suggested the use of *N*/60 sodium or lithium hydroxide for dilutions and dextrose-tryptone agar as a satisfactory plating medium.

(*d*) Ice cream

The increasing consumption of ice cream has awakened interest in the value and safety of this article of food. A few epidemics have been traced to it though such cases are rare. But the obviously unsatisfactory conditions under which some supplies are produced and sold have emphasized the urgent need for more rigid control.

The control of ice cream is more difficult than that of milk. More equipment is used, there is greater risk of contamination after pasteurization, the conditions of sale are often less good, and the product itself is less easy to test. These difficulties have been pointed out by Yale & Hickey (40), who reported the results of a bacteriological study of the ice-cream supplies of a small city. For three of the twelve supplies all plate counts on standard agar were consistently less than 100,000 per g., and for eight supplies the average count was below this figure. The general standard was, however, lower than in districts where control had been instituted. At four centres either the gelatine or the colouring matter was heavily infected and the cream was faulty at another. The equipment was in poor condition at two places. They found that "coliform" counts were more sensitive than plate counts in detecting contamination by the ice-cream dippers or the water in which these were kept. Very similar results have been found by others. Usually the bulk of the contamination is introduced after the mix has been pasteurized. The freezing equipment, the flavourings and colouring matters are the most frequent sources of contamination. The sterilization of the latter is difficult, since many are spoiled by heating to high temperatures. The real solution of this problem is careful selection, treatment and storage at the place where they are first handled. This important question was discussed at the 36th Annual Convention of the International Association of Ice Cream Manufacturers (41,42). The distribution of ice cream in bulk to the retailer who sells it in small quantities is another cause of heavy contamination, and the trouble is aggravated when the dipper is placed in water which is infrequently changed. Conditions are improved when the dipper is kept dry and rinsed with running water immediately before use, but distribution in sealed packages would be a greater safeguard. Two papers by Krog & Dougherty (43) and Abele (44) deal specifically with this question, and others by Frandsen (45), Fabian (46,47), Judkins (48), Bardsley (49) and Grumbine & Halliday (50) deal more generally with the essentials of production of a product of high quality.

There is a wide demand for bacteriological control and the adoption of uniform standards. The Committee on Milk and Dairy Products of the American Public Health Association has issued a report (51) dealing with this question in detail. The International Association of Milk Sanitarians has also issued a report (52) on similar lines. Discussions of bacteriological standards are found in papers by Abele (44), Bardsley (49), Grimes (53), Thomas *et al.* (54), Humphriss (55), Gundel (56), and Sommer (57). There seems to be general agreement that the bacterial count should be less than 200,000 per g. This standard is usually attained even where there is no control, if care is taken in the manufacture. In the U.S.A. twelve states have imposed standards, usually 100,000 per g., although 30,000 per g. has been suggested as not being too severe. Standard meat-extract agar, formerly officially recognized by the American Public Health Association, is an unsatisfactory medium for plate counts. The addition

of skim milk improves the medium, but most American workers appeared to find tryptone-glucose-skim milk agar so satisfactory that this medium is now specified in American Standard methods. Incubation at 32° C. gave much higher counts than incubation at 37° C.(53,58,59).

The "presumptive *coli*" test is widely used in the control of ice-cream manufacture. Stoldt(60) considers that bacterial counts and the "*coli* test" are of no special value, but this is scarcely the general view. The presence of coliform organisms is an indication of post-pasteurization contamination either in the freezer, from added flavouring, etc., or by subsequent handling. It is only rarely that coliform organisms survive the pasteurization process even though the heat resistance of these bacteria is greater in ice cream than in milk. There is, moreover, a tendency to raise pasteurizing temperatures in accordance with new processing methods, and temperatures of 150–160° F. for 30 min. are now used. A positive "*coli* test" cannot therefore be taken as direct evidence of inefficient pasteurization of the mix, it is rather evidence of defective methods of post-pasteurization treatment. The absence of coliform organisms in 0·1 ml. has been suggested as a standard. This question was discussed at the 36th Annual Convention of the International Association of Ice Cream Manufacturers(61).

The use of the phosphatase test for estimating the efficiency of pasteurization of ice-cream mix has been studied by Hahn & Tracy(62), who state that, subject to certain limitations, it may be used for this purpose.

Packing ice cream in paper containers at the plant would avoid the contamination due to the handling of unwrapped bulk by the retailers. In this case, as Abele(44) has pointed out, contamination of the materials of which the cartons were made would be an important factor. Speck & Black(63) have examined paper cartons for contamination and found that while the manufacturers may be responsible for some of the contamination the bulk of it is introduced during storage at the factory or at the retailer's premises (see also in this connexion, p. 517).

It is well known in dairy science circles that what is applicable in one country is not always satisfactory in another, so that adaptation of method, which results in many publications, is often necessary. There is also need for testing theoretical conclusions based on well-supported facts, but mere repetition for the sake of covering paper is to be deprecated. In the course of compiling this review we have encountered the same material many times, and we feel that in these days of universal library service the practice of repeated publication in the same or in different languages should be discouraged.

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